

# **Methods in Hormone Research**

**VOLUME I  
Chemical Determinations**

# **METHODS IN HORMONE RESEARCH**

**Volume I: Chemical Determinations**

**Volume II: Bioassay**



# Methods in Hormone Research

*Edited by*  
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VOLUME I  
**Chemical Determinations**

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## PREFACE

Progress in any scientific field is highly dependent upon developments in methodology. Endocrinology has been a good example of this phenomenon. During the past ten years analytical procedures for the assessment of hormones and their metabolites in body fluids have been improved enormously. Methods have been developed which are capable of separating fractions of a microgram of material and which permit quantitative determination of these important constituents with excellent accuracy. Such developments have led to the first rational hypothesis of certain forms of virilism, have permitted the elucidation of the biochemistry of certain congenital defects of the adrenal, and have been of important diagnostic aid to the clinician.

This volume, the first of a new series on "Methods in Hormone Research," presents for the specialist and student an authoritative review of chemical methods. For the investigator, complete discussions of methodology are presented in a manner sufficiently provocative and critical to stimulate new ideas on the development of yet better methods. Workers in hospital and pharmaceutical laboratories will find discussions and working descriptions in many instances of tested, accurate, sensitive, and convenient methods for repetitive work. This volume should be particularly valuable for the physician who depends on many of these methods as aids in diagnosis and prognosis. For him this volume is a guide to newer and better patient care and is certainly indispensable for his clinical research program.

For the editor, this work has been a great joy due to the fine cooperative spirit of the contributors who are really responsible for the success of the effort. These distinguished colleagues have given liberally of their time so that many may profit.

To Mrs. Iola Graton and Miss Elaine Massad, I am grateful for their devoted cooperation in many technical matters.

RALPH I. DORFMAN

*Shrewsbury, Massachusetts*



# CONTENTS

CONTRIBUTORS TO VOLUME I . . . . .	v
PREFACE . . . . .	vii
CONTENTS, VOLUME II . . . . .	xiii

## 1. Estrogens

JOHN R. K. PREEDY . . . . .	1
I. Introduction . . . . .	1
II. Problems and Difficulties . . . . .	3
III. Criteria for the Assessment of Methods and Procedures . . . . .	4
IV. The Estimation of Estrone, Estradiol-17 $\beta$ , and Estriol: General Procedures . . . . .	4
V. The Use of Isotopes . . . . .	22
VI. Further Identification Procedures . . . . .	25
VII. The Estimation of Conjugates . . . . .	30
VIII. The Estimation of Estrogens in Urine . . . . .	34
IX. The Estimation of Estrogens in Blood, Tissues and Bile . . . . .	41
X. The Problem of Protein Binding . . . . .	44
XI. The Estimation of the Newer Estrogens . . . . .	45
XII. Discussion . . . . .	47
References . . . . .	47

## 2. Assay of 17-Ketosteroids and Testosterone

RALPH I. DORFMAN . . . . .	51
I. Introduction . . . . .	52
II. Determination of Dehydroepiandrosterone . . . . .	53
III. Determination of 5 $\alpha$ -Androst-16-en-3 $\alpha$ -ol (Brooksbank and Haslewood, 1960) . . . . .	59
IV. Determination of Testosterone in Plasma (Finkelstein et al., 1961a) . . . . .	60
V. Determination of 17-Ketosteroids in Urine . . . . .	63
VI. Fractionation of Individual 17-Ketosteroids . . . . .	72
VII. 17-Ketosteroids in Plasma and Serum . . . . .	80
VIII. The Zimmermann Reaction . . . . .	84
IX. Preparation of Neutral Ketonic Fractions . . . . .	85
X. Preparation of the $\beta$ -17-Ketosteroid Fraction . . . . .	86
XI. Miscellaneous Methods . . . . .	88
References . . . . .	88

## 3. Progesterone

JOSEF ZANDER . . . . .	91
I. Historical Survey . . . . .	91
II. Extraction and Preliminary Purification . . . . .	96
III. Isolation of Progesterone from Purified Extracts . . . . .	100
IV. Quantitative Estimation of Isolated Progesterone . . . . .	106
V. Additional Identification of Progesterone . . . . .	115
VI. Reliability of Different Methods . . . . .	118

## CONTENTS

VII. Description of Recommended Methods . . . . .	123
VIII. Application of Methods . . . . .	130
IX. Suggestions for Further Development of Methods . . . . .	133
References . . . . .	134

### 4. Pregnanediol and Pregnanetriol

ARNOLD I. KLOPPER . . . . .	139
I. The Metabolic Precursors of Pregnanediol and of Pregnanetriol . . . . .	140
II. Organs Producing Pregnanediol and Pregnanetriol Precursors . . . . .	142
III. The Occurrence of Pregnanediol and Pregnanetriol . . . . .	144
IV. Quantitative Relationships between Hormone Production and Metabolite Excretion . . . . .	146
V. The Development of Methods for the Assay of Pregnanediol and Pregnanetriol in Urine . . . . .	147
VI. A Critical Evaluation of the Principles of Published Methods of Assay . . . . .	151
VII. Recommended Methods . . . . .	156
VIII. The Clinical Applications of the Assay of Pregnanediol and Pregnanetriol . . . . .	161
IX. Future Developments in the Assay of Pregnanediol and Pregnanetriol . . . . .	165
References . . . . .	167

### 5. Pregnanetriolone, an Abnormal Urinary Steroid

MICHAEL FINKELSTEIN . . . . .	169
I. Introduction . . . . .	169
II. Historical . . . . .	170
III. Methods for the Assay of Pregnanetriolone and Related Steroids in Urine . . . . .	171
IV. Preparation of the Urinary Extracts (Methods I and II) According to Cox and Finkelstein (1957) . . . . .	173
V. Method Ia (Finkelstein, 1959) . . . . .	176
VI. Methods I and Ia: Quantitative Estimation by Fluorometry (Zondek and Finkelstein, 1952; Finkelstein and Goldberg, 1957) . . . . .	177
VII. Method II: Quantitative Estimation through the Determination of Acetaldehyde ("Acetaldehydegenic" Steroids) (Cox, 1959) . . . . .	179
VIII. Other Methods . . . . .	180
IX. Occurrence of Pregnanetriolone . . . . .	180
X. Cases Where Pregnanetriolone Was Not Found (Table II) . . . . .	180
XI. Conditions in Which Pregnanetriolone Is Excreted . . . . .	186
XII. Biosynthesis of Pregnanetriolone . . . . .	193
References . . . . .	196

### 6. Adrenocorticosteroids

FERNAND G. PÉRON . . . . .	199
I. Introduction . . . . .	199
II. Requirements for a Good Chemical Assay . . . . .	201
III. Methods of Assay . . . . .	215
References . . . . .	257



**7. Assay of Aldosterone and Metabolites**

S. A. S. TAIT AND J. F. TAIT . . . . .	265
I. Biological Assay . . . . .	266
II. Physicochemical Assay . . . . .	276
III. Methods of Purification . . . . .	293
IV. Metabolism of Aldosterone in Man . . . . .	300
V. Urinary Assay Methods . . . . .	305
VI. Indirect Secretion Rate Estimates in Man . . . . .	318
VII. Assay in Peripheral Blood . . . . .	323
VIII. Assays of the Adrenal Gland . . . . .	327
References . . . . .	331

**8. Adrenaline and Noradrenaline**

FRED ELMADJIAN . . . . .	337
I. Introduction . . . . .	337
II. Chemistry . . . . .	337
III. Colorimetric Methods . . . . .	341
IV. Fluorometric Methods . . . . .	344
References . . . . .	347

**9. Chemical Assay of Thyroxine-like Materials**

S. B. BARKER . . . . .	351
I. Introduction . . . . .	351
II. Separation of Iodo-Compounds . . . . .	354
III. Chemical Methods of Determination . . . . .	369
IV. Physical Methods of Determination . . . . .	378
V. Summary . . . . .	382
References . . . . .	382

AUTHOR INDEX . . . . .	387
SUBJECT INDEX . . . . .	403



# CONTENTS OF VOLUME II—BIOASSAY

## *Part I: Statistical Methods*

Statistical Methods — C. W. EMMENS

## *Part II: Steroid Hormones and Related Substances*

Estrogens — C. W. EMMENS

Anti-Estrogenic Compounds — RALPH I. DORFMAN

Progestational Substances — TAMOTSU MIYAKE

Anti-Gonadotropic Steroids, Inhibition of Ovulation and Mating  
— ELVA G. SHIPLEY

Androgens and Anabolic Agents — RALPH I. DORFMAN

Anti-Androgenic Substances — RALPH I. DORFMAN

Corticoids — RALPH I. DORFMAN

## *Part III: Protein Hormones and Related Hormones and Substances*

Epinephrine and Norepinephrine — FRED ELMADJIAN

Thyroidal Substances — C. W. TURNER AND B. N. PREMACHANDRA

Insulin — K. L. SMITH

Glucagon — WILLIAM W. BROMER AND OTTO K. BEHRENS

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Melanophore-Expanding Activity — F. W. LANDGREBE AND H. WARING

Relaxin — BERNARD G. STEINETZ, VIVIAN L. BEACH, AND ROBERT L. KROC

The Gonadotropins — ALBERT SEGALOFF

Prolactin — ALBERT SEGALOFF

Thyrotropic Hormone — C. W. TURNER

Adrenocorticotropin — JOSEPH FISHER

Hypophyseal Growth Hormone — HAROLD PAPKOFF AND CHOH HAO LI

## *Part IV: Standard Methods*

Standard Methods Adopted by Official Organizations

— RALPH I. DORFMAN

AUTHOR INDEX

SUBJECT INDEX



## Chapter 1

# Estrogens

JOHN R. K. PREEDY

I. Introduction	1
II. Problems and Difficulties	3
III. Criteria for the Assessment of Methods and Procedures	4
IV. The Estimation of Estrone, Estradiol-17 $\beta$ , and Estriol:	
General Procedures	4
A. Collection and Storage of Samples	4
B. Hydrolysis	5
C. Extraction	7
D. Additional Purification Steps	9
E. Chromatographic Separation	9
F. Quantitative Estimation	18
V. The Use of Isotopes	22
A. Identification of Curves following Column Partition Chromatography	23
B. Estimation of Losses during Hydrolysis, Extraction, and Other Manipulations	23
C. Isotope Dilution	24
D. Reverse Isotope Dilution	25
VI. Further Identification Procedures	25
VII. The Estimation of Conjugates	30
A. Solvent Partition	30
B. Differential Hydrolysis	31
C. Chromatographic Separation	32
D. Quantitative Estimation	34
VIII. The Estimation of Estrogens in Urine	34
A. Methods for Routine Analysis	35
B. Methods Designed Primarily to Identify Urinary Estrogens	40
IX. The Estimation of Estrogens in Blood, Tissues, and Bile	41
A. Plasma	41
B. Tissues, Red Cells, Bile, and Other Material	42
X. The Problem of Protein Binding	44
XI. The Estimation of the Newer Estrogens	45
XII. Discussion	47
References	47

## I. Introduction

The estrogens constitute a group of hormones, which, with other hormones, are responsible for the development and maintenance of the female sex organs and secondary sex characters, and for the maintenance

of the menstrual cycle and pregnancy. In the female the organs of origin are the ovary, the placenta, and the adrenal cortex, and in the male the testicle and the adrenal cortex.

The naturally occurring estrogens are steroids, having an 18- or 19-carbon molecule, an aromatic ring A, and a hydroxyl group at carbon atom 3. The hydroxyl group is therefore phenolic.

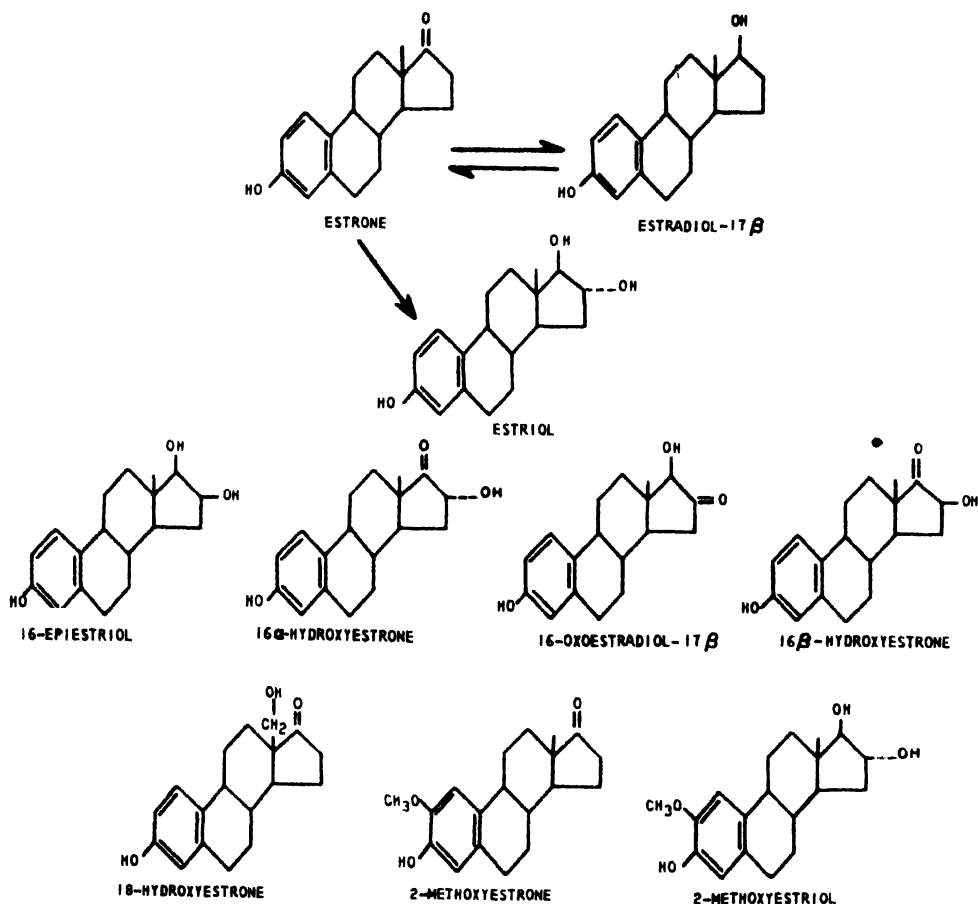


FIG. 1. Above, structure of the three classic estrogens, estrone, estradiol-17 $\beta$ , and estriol, established metabolic pathways being indicated by arrows. Below, structure of some newly discovered human estrogens.

In the human, the three main or "classic" estrogens are estrone, estradiol-17 $\beta$ , and estriol. These were isolated, respectively, in 1929 (Doisy *et al.*; Butenandt), in 1935 (MacCorquodale *et al.*), and in 1930 (Marrian). For many years they were thought to be the only estrogens occurring in the human, although the mono and didehydro derivatives of estrone, equilin, and equilenin, had been found in animals (Girard *et al.*,

1932). The chemical structure of these hormones, together with some of the metabolic transformations known to occur, are given in Fig. 1.

No additional estrogens from human sources were discovered between 1935 and 1955. In the latter year Marrian and Bauld (1955) identified 16-epiestriol (estriol-16 $\beta$ , 17 $\beta$ ) in human pregnancy urine. Further studies in pregnancy urine by Marrian and co-workers resulted in the isolation of 16 $\alpha$ -hydroxyestrone, 16-oxoestradiol-17 $\beta$  and 16 $\beta$ -hydroxyestrone (Layne and Marrian, 1958), and 18-hydroxyestrone (Loke *et al.*, 1959). A further estrogen of somewhat unusual structure, 2-methoxyestrone, was isolated from human urine, following the administration of estradiol-17 $\beta$ -16-C<sup>14</sup> by Kraychy and Gallagher (1957), and by Engel and co-workers (1957). More recently, 2-methoxyestriol was isolated from human urine by J. Fishman and Gallagher (1958). The chemical structure of these newer estrogens are given in Fig. 1.

## II. Problems and Difficulties

Estrogens, in common with other steroids, can exist in the organism in the free state or in conjugation with sulfuric acid or with  $\beta$ -glucuronic acid. It is also possible that additional conjugate forms exist. Both the unconjugated steroids and the conjugates can theoretically be bound to tissue protein, plasma protein, red cell protein, or red cell envelopes, and there is evidence that such estrogen binding occurs biologically, at least in the case of the plasma proteins (see Section X). Estrogens have been demonstrated in human and animal tissues, plasma, red cells, feces, bile, and urine. The isolation of an increasing number of naturally occurring estrogens, the different physicochemical forms in which estrogens may exist, and the variety of tissues and biological fluids in which estrogens are found combine to make a complete analysis of estrogens a formidable problem.

There are however further difficulties. The quantity of estrogen present in most tissues and biological fluids is usually small (of the order of a few micrograms), and the amount of interfering material is frequently large. Available methods for the detection and quantitative estimation of estrogens are in general nonspecific, and procedures used for the preliminary separation of estrogens from interfering material may be inadequate for the purpose. The increasing number of estrogens isolated from natural sources has already been noted. These estrogens have each to be separated from interfering material, as well as from one another. There is as yet a notable lack of methods for the direct estimation of steroid conjugates in general and of estrogen conjugates in par-

ticular. Difficulties in estimating the degree and nature of protein binding are well known; and finally the methods for liberation of estrogens from the protein bond are probably not always quantitative.

In view of these difficulties, it is clear that the various methods and procedures currently available for the estimation of estrogens must be viewed critically, and methods chosen which are considered adequate for a particular purpose, in so far as this is possible. The limitations and imperfections of the methods used should be clearly recognized in the interpretation of the data obtained.

### III. Criteria for the Assessment of Methods and Procedures

In order to assess the validity of methods and procedures, use must be made of appropriate criteria. Suitable criteria have been suggested by Borth (1952), by means of which validity is assessed in terms of accuracy, precision, sensitivity, and specificity. These criteria will be applied to the various procedures described in this chapter.

The definitions of these criteria are as follows: *accuracy*, the extent to which the appropriate added substance can be recovered; *precision*, the degree of agreement of replicate estimations; *sensitivity*, the least quantity of the substance which can be measured; *specificity*, the extent to which the procedure measures only the substance in question. Of these, a high degree of specificity is the most difficult to achieve, although sensitivity is often a problem. Accuracy and precision are more easily achieved, and several procedures are adequate in this respect.

### IV. The Estimation of Estrone, Estradiol-17 $\beta$ , and Estriol: General Procedures

Most of the procedures published relate to the estimation of the classic estrogens: estrone, estradiol-17 $\beta$ , and estriol, and in this section reference will be made only to methods for estimating these three. Methods for estimating the newer estrogens will be discussed in Section XI.

#### A. COLLECTION AND STORAGE OF SAMPLES

Urine can be preserved by adding 5 ml. 50% (v/v) sulfuric acid to the container before collection of a 24-hour sample. Estrogens will remain stable in urine so treated for 1 to 2 weeks at room temperature, although



presumably a minor degree of hydrolysis may occur. If longer storage is necessary, the urine should be stored at  $-15^{\circ}$ . Leon *et al.* (1959) found that urine samples stored at room temperature without preservative for several weeks underwent hydrolysis, and, in addition, the total content of estrone, estradiol- $17\beta$ , and estriol appeared to increase. Blood samples should be centrifuged immediately after collection and the plasma stored at  $-15^{\circ}$ . Plasma estrogens are stable for at least a month under these conditions. With other biological fluids and with tissues, storage at  $-15^{\circ}$  also appears to be the best method for preservation.

## B. HYDROLYSIS

It is probable that virtually all estrogens in human urine are in the form of conjugates. Since methods for the direct estimation of estrogen conjugates are not currently available, it follows that hydrolysis is a necessary preliminary step in the estimation of total urinary estrogens. In plasma, available evidence suggests that estrogens exist in both unconjugated and conjugated forms, and here hydrolysis is again necessary to provide an estimate of total blood estrogens. This also applies to tissues and to other biological fluids.

The classic procedure for the hydrolysis of estrogen conjugates in biological fluids is to boil the fluid with a strong acid. Hydrochloric or sulfuric acid is added to the fluid to be hydrolyzed, and the solution is then refluxed for periods of 30 to 120 minutes. Various recommendations regarding the amount of acid to be added and the length of time of refluxing have been put forward. A recent and authoritative study of the optimal conditions for acid hydrolysis of urine have been made by Brown and Blair (1958). These authors investigated the maximal estrogen yields obtainable from urine under varying conditions, in terms of Brown's method for estimating urinary estrogens (Brown, 1955). Maximal yields were obtained when 15–20 volumes of concentrated hydrochloric acid were added to 100 volumes of urine, and the solution refluxed for 60 to 120 minutes. Within these limits the yields of estrogen remained approximately constant. The amount of acid added was inversely proportional to the refluxing time required. The authors were also able to show that hydrolysis by this procedure was accompanied by some destruction of the estrogens.

These findings have been briefly confirmed in the author's laboratory. In the method of Precdy and Aitken (1961b), an aliquot of urine is brought to the boil, and concentrated HCl is added down the refluxing condenser in the proportion 15 ml. acid to 85 ml. urine; the resulting mix-

ture is refluxed for 45 minutes. This procedure is in accord with the recommendations of Brown and Blair (1958), and has been found to give a maximal yield.

Acid hydrolysis as described above has also been used by Preedy and Aitken (1961b) in the determination of plasma estrogens. Here acid hydrolysis has the combined effect of hydrolyzing conjugates and denaturing the plasma proteins, with presumed quantitative release of estrogens from the protein bond. This procedure will be discussed further in Sections IX and X.

Continuous extraction for 48 hours or more with ether or chloroform from an acid pII (usually 1.0 or less) has been used by various authors for the hydrolysis of urine, blood, and tissue estrogen conjugates (Slaunwhite and Sandberg, 1956; Hagopian and Levy, 1958; Migeon *et al.*, 1959; Beer and Gallagher, 1955a). This is a somewhat less drastic procedure than conventional acid hydrolysis, and presumably results in less destruction of estrogens although comparative studies have not been published. This form of hydrolysis is considered to result in quantitative cleavage of estrogen sulfates, but not of estrogen glucosiduronates.

In general, sulfates appear to be more easily hydrolyzed than glucosiduronates, and a very mild treatment probably suffices. S. L. Cohen and Oneson (1953) found that various ketosteroid sulfates, including estrone sulfate, could be hydrolyzed quantitatively by allowing them to stand overnight in 1 to 10 ml. of 1,4-dioxane at room temperature. Glucosiduronates were not affected by this treatment. Burstein and Lieberman (1958) were able to show that various ketosteroid sulfates could be similarly hydrolyzed by shaking them with ether or ethyl acetate under appropriate conditions. The use of differential hydrolysis procedures in the estimation of estrogen conjugates will be discussed in Section VII.

Enzymatic hydrolysis has been used to an increasing extent in the estimation of total estrogens, particularly in urine. Material obtained from bacterial sources and from organs of animals, shellfish, and snails may contain large amounts of  $\beta$ -glucuronidases and/or phenolsulfatases capable of hydrolyzing estrogen conjugates. The beef-liver preparation Ketodase ® (Warner-Chilcott Company) has been most frequently employed for hydrolyzing glucosiduronates (Beer and Gallagher, 1955a, b; Gallagher *et al.*, 1958; Dancis *et al.*, 1958; Migeon *et al.*, 1959). Urine is adjusted to pH 4.7–5.0 with acetate buffer, 300–500 Fishman units of Ketodase per milliliter of urine are added, and the mixture is incubated at 37° for 5 days. Although shorter periods (e.g., 48 hours) have been employed, there is some doubt whether estriol glucosiduronate is quantitatively hydrolyzed in less than 5 days.

Acetone-dried material from the gut of the limpet (*Patella vulgata*) contains both glucuronidases and sulfatases, and has been used successfully by Brown and Blair (1958) for the hydrolysis of estrogen conjugates in urine. The material is added to the urine, the pH is adjusted to 4.7 using acetate buffer, and the mixture is incubated at 37° for 96 hours. Very satisfactory results were obtained if a minimum of 600 units glucuronidase and over 2500 units sulfatase per milliliter urine were used. A higher yield was obtained with enzyme hydrolysis than with acid hydrolysis, and the enzyme hydrolysis was considered to be quantitative.

A preparation containing phenolsulfatase, Mylase (Wallerstein Laboratories), is available, and has been used to hydrolyze estrogen sulfates; 200 mg. are added per 100 ml. of urine, the pH is adjusted to 6.0, and the mixture incubated at 50°C. for 24 hours under a layer of toluene (H. Cohen and Bates, 1949). Under these conditions, hydrolysis of sulfates is thought to be quantitative (Cohen and Bates, 1949; Dancis *et al.*, 1958).

There are various advantages in using enzymatic hydrolysis. Firstly, the treatment is mild, compared with acid hydrolysis, and no significant destruction of estrogen occurs, as indicated by Brown and Blair (1958). Secondly, acid hydrolysis itself tends to produce much interfering material. This does not occur to the same extent with enzymatic hydrolysis. Thirdly, many of the newer estrogens are unstable when refluxed with strong acid, and enzymatic hydrolysis may then be the only form of hydrolysis which can be used.

On the other hand, enzyme hydrolysis is, in general, expensive and time consuming. Although conjugates of estrone and estradiol-17 $\beta$  are readily hydrolyzed in 24 hours, those of estriol appear to require up to 5 days. Brown and Blair (1958), using the method of Brown (1955), found that with enzyme-treated urine, although material interfering with the estimation of estrone and estradiol-17 $\beta$  was reduced (as compared with acid-treated urine), material interfering with the estimation of estriol was actually increased. A similar situation has been experienced using the method of Preedy and Aitken (1961b).

### C. EXTRACTION

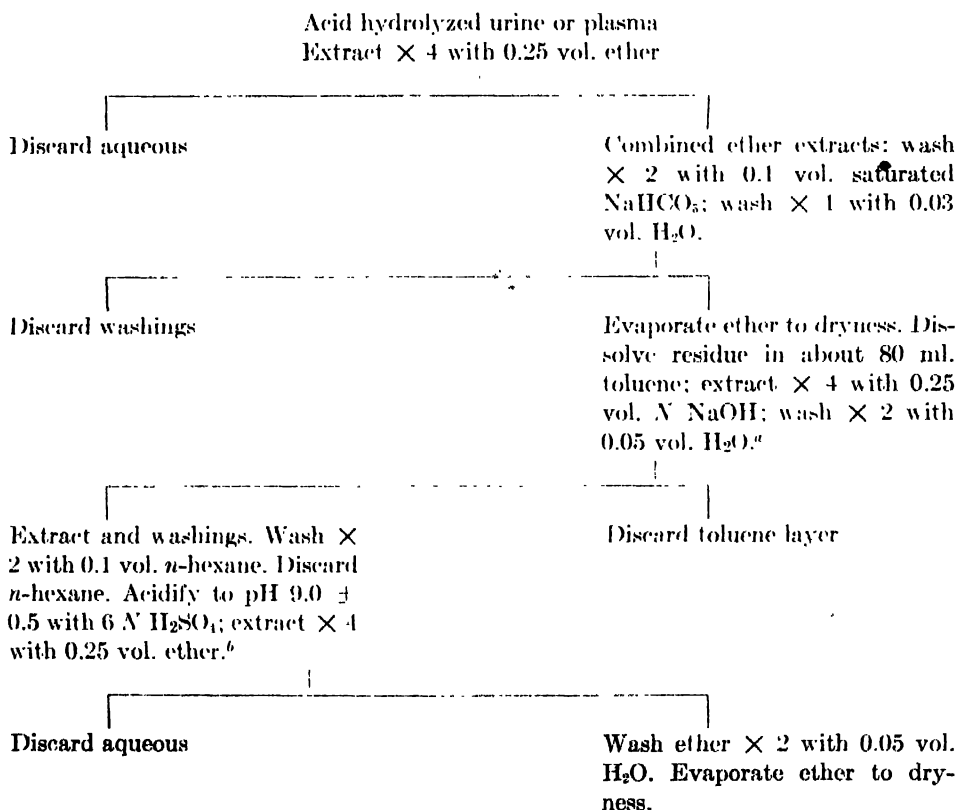
After hydrolysis of conjugates in any biological fluid, the free estrogens so released must undergo concentration and preliminary purification by a series of hand extractions. The design of a suitable extraction procedure depends on a consideration of estrogen partition coefficients between various immiscible solvent pairs. Two extraction procedures have been generally applied to urine, those of Engel *et al.* (1950) and of

Brown (1955). Both procedures depend on a preliminary extraction of the hydrolyzed urine with ether; a phenolic partition between NaOH and an organic solvent such as toluene, benzene, or light petroleum, in which the estrogens, being phenolic, remain in the alkaline phase, and neutral lipids enter the organic phase; and an ether extraction from an aqueous phase adjusted to pH 9.0–10.0. Many variants of these procedures have been used.

The method of Brown (1955) includes in addition a partition between benzene-light petroleum (1:1) and water. In this partition, estriol is quantitatively separated from estrone and estradiol-17 $\beta$ , the former being distributed only in the aqueous phase and the latter two only in

TABLE I

EXTRACTION PROCEDURE FOR URINARY OR PLASMA ESTROGENS (Engel *et al.*, 1950)



<sup>a</sup> Dried ether extract should be dissolved in 1 N NaOH, as well as in toluene, since estriol is virtually insoluble in toluene; 200 ml. toluene should be used in extracting plasma.

<sup>b</sup> A buffer containing 20 vols. saturated KHCO<sub>3</sub> and 1 vol. saturated K<sub>2</sub>CO<sub>3</sub> is useful when adjusting to pH 9.0.

the organic phase. A partition between water and benzene appears to be equally effective (West *et al.*, 1958a). Separation of estriol from estrone and estradiol-17 $\beta$  is not performed in the extraction procedure of Engel *et al.* (1950).

The procedure of Engel *et al.* (1950) has the advantage that only one extraction sequence is involved, as opposed to two sequences in those methods employing the water-benzene partition. It is simple to use, and the loss of estrone, estradiol-17 $\beta$ , and estriol during processing is less than 5%. It has been found most satisfactory in the author's laboratory, where it has been used for a number of years. It has been incorporated into the method of Precdy and Aitken (1961b), and is given in Table I. It can be applied to plasma as well as to urine.

#### D ADDITIONAL PURIFICATION STEPS

Even after the procedures described above, the resulting extract tends to be bulky and contains much interfering material, particularly in the case of urine. Consequently, any further steps which can be taken to reduce the interfering material will be of value, and two additional purification steps are worth mentioning. It has been found that the three classic estrogens are resistant to boiling in dilute aqueous alkali and that this greatly reduces the amount of material interfering with the measurement of estrogens by the Kober method. This additional step was described by Bauld (1956), and has been incorporated into the method of Brown for urinary estrogens (Brown, 1955; Brown *et al.*, 1957b).

Theoretically, ion exchange resins should be valuable in the further purification of urinary extracts. Engel (1958) has described the use of Dowex 2 anion exchange resin columns, in the bicarbonate form. Phenolic (or total) extracts are dissolved in methanol and transferred to the column. About 70 ml. of methanol is allowed to flow through the column and the estrogens are then eluted using 3 to 5 *N* acetic acid in methanol. Evolution of CO<sub>2</sub> is disregarded. An 80% reduction in interfering fluorescent material is claimed. A description of this method will soon be published. It should prove of considerable value.

#### E. CHROMATOGRAPHIC SEPARATION

Methods for the quantitative estimation of estrogens cannot be applied directly to crude extracts of urine, plasma, or other biological material, due to the presence of the large amounts of interfering substances already referred to. Further separation of estrogens is essential. It was at this stage that methodology for the chemical estimation of estrogens was

held up for many years for lack of appropriate techniques. The advent of chromatography made such separation possible for the first time.

Several chromatographic techniques have been applied to the estimation of estrogens in biological fluids, particularly in urine: paper chromatography; adsorption column chromatography; ion exchange chromatography; countercurrent distribution and column partition chromatography; and gas chromatography.

### *1. Paper Chromatography*

In the simplest form of this type of chromatography, the crude material is placed at one end of a length of filter paper in a small volume of solvent, which is then allowed to dry. The paper is then suspended from a trough, the whole being enclosed in a glass tank. A solvent or solvent mixture is placed in the trough and is allowed to flow slowly down the length of the paper. The various substances contained in the crude material applied to the paper move downward from the point of origin at varying speeds, depending on the affinity of each for the paper (or more accurately the paper-water complex) on the one hand, and the solvent on the other. The ratio of the distances from the point of origin of a substance and of the solvent front is known as the  $R_f$  value. Substances with a high  $R_f$  value (e.g., 0.9) move near the solvent front, and those with a low  $R_f$  value (e.g., 0.1) move more slowly from the point of origin.

Paper chromatography depending on the use of one solvent phase as described has a limited application only. The use of two solvent phases greatly increases versatility, and this form of paper chromatography is generally used. The paper is allowed to take up an appropriate solvent (usually water miscible), which is then regarded as the stationary phase, and a second solvent (usually an organic solvent, which is immiscible with water and with the stationary phase) is allowed to flow over the paper. This second solvent is the mobile phase. The paper then acts mainly as a supporting medium for the stationary phase, although it probably still retains adsorptive properties to some degree. The composition of both stationary and mobile phases can of course be varied, making this a versatile method of chromatography.

Paper chromatography has been used in estrogen estimations by a number of workers. A summary of the solvent systems used for each of the three classic estrogens, together with the appropriate references, is given in Table II.

The advantages of paper chromatography in the estimation of estrogens are as follows: (a) it is relatively easy to perform, little apparatus being required; (b) it is relatively sensitive, homogeneous material being concentrated at one spot, rather than distributed over a series of frac-

**TABLE II**  
**PAPER CHROMATOGRAPHY OF ESTROGENS**

Estrogen	Solvent systems		$R_f$ value	Reference
	Stationary phase	Mobile phase		
Estrone	Formamide	<i>o</i> -Dichlorobenzene		Axelrod (1953)
Estradiol-17 $\beta$	Formamide	<i>o</i> -Dichlorobenzene		Axelrod (1953)
Estradiol-17 $\beta$	Formamide	Cyclohexane		Axelrod (1953)
Estriol	Formamide	Methylene chloride		Axelrod (1953)
Estrone	Methanol	Petroleum ether		Mitchell and Davies (1954)
Estradiol-17 $\beta$	Methanol	Petroleum ether		Mitchell and Davies (1954)
Estriol	Methanol 50% Water 50%	Benzene		Mitchell and Davies (1954)
Estrone	Methanol 80% Water 20%	Benzene 33% Petroleum ether 67%	0.33	Slaunwhite and Sandberg (1956)
Estriol	Formamide	Chloroform		Dancis <i>et al.</i> (1958) Levitz <i>et al.</i> (1956a,b)
Estradiol-17 $\beta$	Formamide	Benzene		Levitz <i>et al.</i> (1958)
Estrone	Formamide	Monochlorobenzene	0.68	Breuer and Nocke (1958)
Estradiol-17 $\beta$	Formamide	Monochlorobenzene	0.25	Breuer and Nocke (1958)
Estrone	Formamide	Chloroform	0.89	Breuer and Nocke (1958)
Estradiol-17 $\beta$	Formamide	Chloroform	0.65	Breuer and Nocke (1958)
Estriol	Formamide	Chloroform	0.05	Breuer and Nocke (1958)
Estriol	Methanol 80% Water 20%	Isooctane 25% Toluene 75%	0.02	Migeon <i>et al.</i> (1959)
Estradiol-17 $\beta$	Methanol 80% Water 20%	Isooctane 25% Toluene 75%	0.54	Migeon <i>et al.</i> (1959)
Estrone	Methanol 80% Water 20%	Isooctane 25% Toluene 75%	0.78	Migeon <i>et al.</i> (1959)
Estriol	Methanol 70% Water 30%	Benzene 40% Petroleum ether 60% [Skelly C] <sup>86</sup>	0	Migeon <i>et al.</i> (1959)
Estradiol-17 $\beta$	Methanol 70% Water 30%	Benzene 40% Petroleum ether 60% [Skelly C] <sup>86</sup>	0.28	Migeon <i>et al.</i> (1959)
Estrone	Methanol 70% Water 30%	Benzene 40% Petroleum ether 60% [Skelly C] <sup>86</sup>	0.65	Migeon <i>et al.</i> (1959)
Estriol	Methanol 55% Water 45%	Benzene	0.09	Migeon <i>et al.</i> (1959)
Estradiol-17 $\beta$	Methanol 55% Water 45%	Benzene	0.79	Migeon <i>et al.</i> (1959)
Estrone	Methanol 55% Water 45%	Benzene	0.88	Migeon <i>et al.</i> (1959)

NOTE: All percentages are v/v, e.g., methanol 70%, water 30%, means 70 parts methanol added to 30 parts water.

tions, as in column partition chromatography; (c) it is particularly suited to rapid qualitative and semiquantitative analysis, although it can of course be used for accurate quantitative analysis, especially in combination with other chromatographic procedures (West *et al.*, 1958a, b; Levitz *et al.*, 1958).

The disadvantages are: (a) large quantities of material cannot be handled, so that phenolic extracts of urine, for instance, cannot in general be chromatographed directly in this way; (b) resolution is frequently not as good as with column partition systems, a degree of "tailing" may occur particularly in the presence of significant amounts of interfering material; (c) the spots are usually somewhat irregular in shape, and it is therefore often difficult to be certain whether a spot is composed of homogeneous material or not; (d) quantitative removal of the spot from the paper may present difficulties in some circumstances, due to the adsorptive qualities of the paper.

## 2. Adsorption Column Chromatography

This procedure resembles that of the simplest form of paper chromatography, described above. The substances to be chromatographed are placed in top of a column of a chemically inert adsorbent material, such as alumina, or silicic acid (silica gel), containing a certain amount of water. An eluent, usually an organic solvent mixture, is then allowed to run through the column. Substances move on the column at a rate depending on their relative affinities for the adsorbent material on the one hand, and for the eluent on the other. Frequently eluents of varying composition are used sequentially (see Table III).

Adsorption column chromatography has been much used in the estimation of estrogens in body fluids and tissues. Alumina columns form an essential part of the method of Brown (1955) for urinary estrogens, and have also been used by Eberlein *et al.* (1958) in their simplified method for urinary estriol. Silica gel columns have been used by Kushinsky *et al.* (1958), Beer and Gallagher (1955a, b), Hagopian and Levy (1958), Sandberg and Slaunwhite (1957), and Levitz *et al.* (1956a, b). A summary of these procedures for separating the three classic estrogens, with the various eluent mixtures employed is given in Table III.

Advantages of these adsorption systems are: (a) fairly large amounts of material can be handled by the columns, thus providing an advantage over paper chromatography; (b) preparation of the column is very simple, a minimum of apparatus being required.

However, there are certain disadvantages in this technique. Standardization of the alumina, and to a lesser extent of the silica gel, in terms of adsorption characteristics is often difficult. One sample may vary



greatly from another in this respect. The moisture content is frequently critical, a small percentage variation greatly altering the characteristics of the adsorbent. Separation of individual substances is much less efficient than with paper or column partition chromatographic systems,

TABLE III  
ADSORPTION COLUMN CHROMATOGRAPHY OF ESTROGENS  
ADSORBENT MATERIAL AND ELUENT SYSTEMS

Estrogen	Adsorbent material	Eluent <sup>a</sup>	Reference
Estrone	Silica gel	Benzene 90%, Ethyl acetate 10%	Levy (1954)
Estradiol-17 $\beta$	Silica gel	Benzene 85%, Ethyl acetate 15%	Levy (1954)
Estrone Estradiol-17 $\beta$	Silica gel	Increasing concentrations of ethyl acetate in petroleum ether	Beer and Gallagher (1955a,b)
Estrone Estradiol-17 $\beta$			
Estrone Estradiol-17 $\beta$	Silica gel	4% Diethyl ether in benzene	Meyer (1955)
Estrone	Florisil	25% Isopropanol in ethyl acetate	Meyer (1955)
Estrone	Florisil	Increasing concentrations (2.0-7.5%) methanol in methylene chloride	Slaunwhite and Sandberg (1956)
Estrone Estradiol-17 $\beta$	Silica gel	2% methanol in benzene	Levitz <i>et al.</i> (1956a,b)
Estrone Estradiol-17 $\beta$			
Estrone	Silica gel	4% methanol in benzene	Levitz <i>et al.</i> (1956a,b)
Estrone	Silica gel	Benzene 50%, Ethyl acetate 50%	Hagopian and Levy (1958)
Estrone Estradiol-17 $\beta$	Silica gel	Increasing concentrations of ethyl acetate in benzene	Kushinsky <i>et al.</i> (1958)
Estrone Estradiol-17 $\beta$			
Estrone	Alumina	30% Ethanol in benzene	Eberlein <i>et al.</i> (1958)
Estrone methyl ether	Alumina	25% Benzene in petroleum ether	Brown (1955)
Estrone methyl ether	Alumina	40% Benzene in petroleum ether	Brown (1955)
Estrone methyl ether	Alumina	2% Ethanol in benzene	Brown (1955)

<sup>a</sup> For significance of percentages, see note to Table II.

for instance, and tailing may be prominent. It is very difficult to be certain that curves derived from fractions obtained from the column represent homogeneous material, since these curves are seldom regular in outline when volume of eluent is plotted against concentration of material. Frequently, relatively large volumes of eluent are bulked. Under these circumstances it is even more difficult to be certain that the material

eluted is homogeneous. Finally it may not always be possible to elute substances quantitatively from the column.

Adsorption chromatography is best employed in estrogen determinations as a preparatory step, where the ability to handle relatively large quantities of material can be used to advantage, to be followed by further chromatography or by some other separatory procedure, as in the studies of Levitz *et al.* (1956a, b, 1958), Slaunwhite and Sandberg (1956), and Levy (1954). It is doubtful whether adsorption chromatography can be used as the sole means for the chromatographic separation of estrogens from urinary interfering material, particularly in any circumstance where the ratio between interfering material and estrogens is high.

### 3. *Ion Exchange Chromatography*

Theoretically ion exchange chromatography should be expected to work well in the separation of estrogens but in practice it has not been much used, apart from the preliminary purification step of Engel (1958) mentioned in Section IV, D. However, Seki (1958), in a preliminary communication, found that estrone, estradiol-17 $\beta$ , and estriol could in fact be separated on specially prepared Amberlite IRC-50 (a carboxylic cation exchange resin), elution being carried out with a mixture of ethanol and 1 N HCl. The degree of separation does not appear to be as good as that obtained with other chromatographic systems, and the difficulty of non-specific adsorption onto the resin may not have been solved.

### 4. *Countercurrent Distribution and Column Partition Chromatography*

Although countercurrent distribution is not usually classed as chromatography, these two methods depend on the same principle and can conveniently be considered together.

The principle makes use of the different partition coefficients of single substances between immiscible solvent pairs. When a single substance is added to a flask containing equal volumes of two immiscible solvents, then, after equilibration, this substance will be distributed between the two solvents in a certain ratio. The ratio of the concentrations in upper and lower solvent layers is the partition coefficient.

This principle of partition can be applied in two different ways, countercurrent distribution and column partition chromatography. (It should be noted that two-phase paper chromatography is also an application of the partition principle.)

In countercurrent distribution, sequential partitions between immiscible solvent pairs are carried out in a series of glass tubes. Aliquots of the heavier of the two solvents are placed in a series of tubes and constitute a type of stationary phase. The crude material to be separated is

added to the first tube. An aliquot of the lighter of the two solvents (which can be regarded as the mobile phase) is added to the first tube, and after thorough equilibration, is transferred to the second tube where the process is repeated. A fresh aliquot of the lighter solvent is then added to the first tube, and the process of transferring the lighter solvent from one tube to the next tube after equilibration continues along the series of tubes. Countercurrent apparatus with automatic equilibration and transfer is available. Any number of tubes or "transfers" from 24 to 240 may be used depending on the application. Countercurrent distribution has been employed in the estimation of estrogens by Engel *et al.* (1950, 1957, 1958), Diczfalusy *et al.* (1957), Gallagher *et al.* (1958), West *et al.* (1958a, b), and others. Solvent systems used in countercurrent distribution of estrogens are given in Table IV. It should be observed that in

TABLE IV  
COUNTERCURRENT DISTRIBUTION OF ESTROGENS

Estrogen <sup>a</sup>	Solvent systems <sup>b</sup>		No. of transfers	Reference
	Solvent I	Solvent II		
Estrone Estradiol-17 $\beta$	Methanol 70% Water 30%	Carbon tetrachloride	99-130	Engel <i>et al.</i> (1957) Gallagher <i>et al.</i> (1958)
Estriol	Ethanol 50% Water 50%	Ethyl acetate 50% Cyclohexane 50%	99	Gallagher <i>et al.</i> (1958)
Estrone	Ethanol 60% Water 40%	Ethyl acetate 40% Hexane 60%	150	West <i>et al.</i> (1958 a, b)
Estradiol-17 $\beta$	Ethanol 60% Water 40%	Ethyl acetate 40% Hexane 60%	200	West <i>et al.</i> (1958 a, b)
Estriol	Ethanol 50% Water 50%	Ethyl acetate 50% Hexane 50%	299	West <i>et al.</i> (1958 a, b)

<sup>a</sup> Partition coefficients for estrone, estradiol-17 $\beta$ , and estriol in numerous solvent systems are given by Engel *et al.* (1950) and by Bauld and Greenway (1957).

<sup>b</sup> For significance of percentages, see note to Table II.

countercurrent distribution, the denser of the two solvents is regarded as the stationary phase, whereas in paper chromatography and partition chromatography the stationary phase is the more polar, or water miscible, of the two solvents. (In reverse phase column chromatography, the opposite is true, the mobile phase being the more polar, or water miscible.) A description of countercurrent distribution is given by Williamson and Craig (1947).

In column partition chromatography, the stationary phase, which must

be miscible with water (e.g., 70% aqueous methanol) is adsorbed onto an inert powder such as kieselguhr (Celite) which then acts as a scaffolding for the stationary phase and is called the supporting phase. The supporting phase together with the stationary phase is packed into a column 10–20 cm. in length, and a mobile phase (e.g., carbon tetrachloride) is allowed to flow through the column. Under these conditions, Celite has virtually no adsorbing effect on the material to be chromatographed, and substances move in the column at a speed depending on their partition coefficients between the mobile and stationary phases, in the same way as in countercurrent distribution. The similarity between this procedure and two-phase paper chromatography will again be apparent.

TABLE V  
COLUMN PARTITION CHROMATOGRAPHY OF ESTROGENS

Estrogen	Solvent systems <sup>a</sup>		Reference
	Stationary phase	Mobile phase	
Estrone	0.8 N NaOH	Benzene	Bauld (1956)
Estradiol-17 $\beta$	0.8 N NaOH	Ethylene dichloride 66% Benzene 34%	Bauld (1956)
Estriol	0.8 N NaOH	Ethylene dichloride	Bauld (1956)
Estrone	Methanol 72% Water 28%	Carbon tetrachloride 20% <i>n</i> -Hexane 80%	Preedy and Aitken (1961b)
Estradiol-17 $\beta$	Methanol 72% Water 28%	Carbon tetrachloride 15% Chloroform 11.2% <i>n</i> -Hexane 73.8%	Preedy and Aitken (1961b)
Estradiol-17 $\beta$	Methanol 72% Water 28%	Carbon tetrachloride 65% <i>n</i> -Hexane 35%	Preedy and Aitken (1961b)
Estriol	Methanol 72% Water 28%	Chloroform 48% <i>n</i> -Hexane 52%	Preedy and Aitken (1961b)

Length of columns 10–15 cm.

<sup>a</sup> For significance of percentages, see note to Table II.

Column partition chromatography has been extensively used in the estimation of estrogens of urine and of plasma by Preedy and Aitken (1961b) and of urine by Bauld (1956). The use of column partition chromatography will be further discussed in Section VIII. A table of solvent systems for column partition chromatography used by various authors in the estimation of the three classic estrogens is given in Table V.

The advantages of both countercurrent distribution and column partition chromatography are considerable. Adsorption is not involved, and

the results of partition chromatography for a given substance, using a given solvent pair, is mathematically predictable from a consideration of known factors, such as partition coefficients, dimensions of column, column volume, number of transfers (Bauld and Greenway, 1957). No precise standardization of materials is required. Reasonably large quantities of material can be handled. Systems can be selected with very high resolving power, and efficient and reproducible separation of individual substances can then be obtained. Single substances are distributed in Gaussian curves, when concentration is plotted against volume of eluent. This latter constitutes an important method of identification, since if a curve is symmetrical and Gaussian in shape, it is likely to be composed of homogeneous material. Nonhomogeneity can usually be recognized by asymmetry of a curve, and in this way the system can be constantly monitored.

Disadvantages of the systems are minor compared with the advantages. The apparatus required is somewhat more elaborate than that in the other forms of chromatography already discussed. The procedure is more time consuming, in that multiple fractions must be obtained and analyzed. There is some loss of sensitivity compared with paper chromatography, since a substance is distributed over several fractions of eluate, rather than appearing as a single spot.

Compared with column partition chromatography, the advantages of countercurrent distribution are: (a) there is no reasonable limit to the amount of material which can be handled; (b) the sequential transfers may be stopped at any time, and the tubes sampled; (c) the solvent system is entirely stable. The one disadvantage is that a large and expensive apparatus is required. Automatic equipment is virtually a necessity, together with 100 to 250 transfer stages. This limits the number of simultaneous estimations that can be performed. It is doubtful whether transfers numbering 24 or less are of much value, except where minimal resolution is required, such as in a preliminary separation procedure, or where there is no significant amount of interfering material.

In column partition chromatography no expensive apparatus is required, a column 15 cm. high having the same resolving power as approximately 180 countercurrent transfers. Consequently, several estimations may be carried out simultaneously. However, column partition chromatography has the disadvantage that, although relatively large amounts of material can be chromatographed, there is a definite upper limit beyond which the column may become unstable. Relatively miscible solvent pairs (e.g., water, butanol) cannot usually be employed due to instability in the column. However, these are not often practical considerations in the

determination of estrogens, and the minor limitations of column partition chromatography are far outweighed by the great advantages of high resolving power.

### *5. Gas Chromatography*

There are two principal forms of gas chromatography, gas adsorption chromatography, and gas partition chromatography. The principles involved are similar, respectively, to those of adsorption column chromatography and column partition chromatography already described, but in each case a gas replaces the liquid as mobile phase, and chromatography takes place at a high temperature.

Although it has been evident for some time that gas chromatography might be of great value in the determination of steroid hormones, technical difficulties, such as instability of steroids and certain steroid derivatives at high temperature, had not until recently been overcome. However, Wotiz and Martin (1961) have now demonstrated that estrone, estradiol-17 $\beta$ , and estriol in the form of acetates may be separated with great ease by gas chromatography.

The sensitivity of the procedure is currently somewhat less than that of sulfuric acid fluorescence, but this can probably be rectified by a more sensitive detection system.

The great advantage of gas chromatography is the rapidity with which good separation of estrogens can be effected. Using silicone grease as stationary phase on a supporting phase of Chromosorb, with helium as mobile phase, Wotiz and Martin (1961) were able to achieve separation of the three estrogens in 45 minutes, with a resolution comparable to that obtained by column partition chromatography after 24 hours.

The application of this procedure to the estimation of estrogens in biological fluids is awaited with interest.

## F. QUANTITATIVE ESTIMATION

Although there are many methods for the detection and quantitative estimation of estrogens, none of them have been shown to be sufficiently specific for the quantitative estimation of estrogens in biological fluids without extensive preliminary purification and separation of the estrogens from interfering material. Consequently, the specificity of a complete method for estrogen determination will in general depend not on the final detection procedure employed but on the efficiency of previous steps taken to eliminate interfering material.

Although the various detection procedures share a lack of specificity, there is a considerable difference in sensitivity. Certain procedures are

relatively insensitive and will not be discussed. These include ultraviolet light adsorption in the 280-m $\mu$  region, and polarography (for estrone). Certain nonspecific color reagents have been used to detect and estimate estrogens in association with paper chromatography. Turnbull's blue [equal quantities of 1% solutions of FeCl<sub>3</sub> and of K<sub>3</sub>Fe (CN)<sub>6</sub>] has proved particularly useful (Dancis *et al.*, 1958; Dao, 1957; Meyer, 1955). Other reagents used are diazotized sulfanilic acid (Wall and Migeon, 1959), and Folin and Ciocalteu's reagent for phenols (Mitchell and Davies, 1954). A list of color reactions for estrogens are given by Axelrod (1953) and Shoppee (1958).

Detection procedures for estrogens in current use fall into the following groups: colorimetric, fluorimetric, and enzymatic.

### 1. Colorimetric

Of the colorimetric reactions, the Kober reaction is best known (Brown, 1955; Bauld, 1956; Diezfalussy and Linkvuist, 1956). Many modifications have been suggested, and that of Bauld (1954) has been the most used. This modification of the Kober reaction was incorporated into Brown's method for estimating urinary estrogens (Brown, 1955). The reaction is performed as follows: the color-producing reagent for estriol is 2% quinol in 76% (v/v) H<sub>2</sub>SO<sub>4</sub>; for estrone, 2% quinol in 66% H<sub>2</sub>SO<sub>4</sub>; for estradiol-17 $\beta$ , 2% quinol in 65% H<sub>2</sub>SO<sub>4</sub>. Three milliliters of the appropriate reagent is added to the dried material, which is then heated in a boiling water bath for 20 minutes and cooled. Depending on the estrogen, 0.2 to 1.0 ml. water is added and after shaking the solution is again heated in the boiling water bath for a further 10 minutes. The intensity of the color produced is measured at 430, 516, and 552 m $\mu$ .

A considerable degree of specificity has been claimed for the Kober reaction at various times. Although it has been of great value in detecting the newer estrogens in late pregnancy urine (Marrian and Bauld, 1955; Marrian *et al.*, 1957; Layne and Marrian, 1958), it is, in common with the other procedures, not of sufficient specificity to justify its use for the estimation of estrogens in the presence of significant interfering material without efficient preliminary separation from such material. It is to be noted in Brown's method (Brown, 1955), that even after preliminary extraction and adsorption chromatography, a considerable spectrophotometric correction has to be applied (the "Allen" correction: Allen, 1950), indicating that much interfering color may be produced during the Kober reaction. The correction (in terms of units of density) may be several times greater than the estrogen reading in low-titer urines (Brown *et al.*, 1957a; Ittrich, 1958). Although more sensitive than some procedures (e.g., ultraviolet absorption), the Kober reaction is much less sensitive

than sulfuric acid fluorimetry, for instance, and can only with difficulty be applied to the measurement of the low concentrations of estrogens which exist in plasma.

A further modification of the Kober reaction has been proposed by Ittrich (1958). The Kober color is extracted into chloroform containing 2% *p*-nitrophenol and 1% ethanol. The color is measured by absorption at 518–538  $m\mu$ , and an amount as low as 0.2  $\mu g$ . estrogen in 2.5 ml. can be measured. A high degree of specificity is claimed, and evidence for its superiority over existing Kober modifications is given.

Of greater interest is the fact that the "Kober material" fluoresces under these circumstances, and sensitivity comparable to that of the sulfuric acid fluorescence procedures (see following section) can then be obtained (Ittrich, 1958). The fluorimetric application of the Ittrich reaction appears to be a valuable addition to the methods for the detection of estrogens.

## 2. Fluorimetric

Estrogens fluoresce strongly when heated with phosphoric or sulfuric acids. Phosphoric acid fluorescence has been recommended by Finkelstein (1952), who claims that although this procedure is less sensitive than sulfuric acid fluorescence, it is also less subject to interference. This method has not found general acceptance.

Sulfuric acid fluorescence, on the other hand, has been extensively used. It was first introduced by Jailer (1948), and modified by Bates and Cohen (1950a, b). The conditions recommended by Bates and Cohen (1950a) have been adopted by Engel *et al.* (1950), and Preedy and Aitken (1961a, b). Optimal spectrophotometric conditions for the measurement of various estrogens by sulfuric acid fluorescence have been investigated by Slaunwhite *et al.* (1953), Goldzieher (1953), Aitken and Preedy (1953), and Bauld *et al.* (1960).

Optimal conditions for estimating urinary estrogens by sulfuric acid fluorescence, following column partition chromatography, are given by Preedy and Aitken (1961a, b). According to the recommendations of the latter authors, the estrogen-containing material is dissolved in a 0.1-ml. benzene-ethanol mixture (19 parts benzene–1 part 95% ethanol), 0.2 ml. 90% (v/v) sulfuric acid is added, and the mixture heated at 80° for 10 minutes. After cooling, 1.4 ml. 65% (v/v) sulfuric acid is added, and the mixture is allowed to stand for 1 hour. These conditions correspond to those recommended by Engel *et al.* (1950). Fluorimetry is then carried out, using the 405- and 436- $m\mu$  Hg lines as exciting light, but excluding the 350- $m\mu$  line, and reading the fluorescence emission at 490  $m\mu$ .



The sulfuric acid method is distinguished by extreme sensitivity. By using the recommended optical conditions and reagents, the amount of each of the three classic estrogens which can be estimated is limited chiefly by the fluorescence of the reagent blank. The blank should have a fluorescence intensity equivalent to less than 0.001  $\mu\text{g.}$  estrone. Therefore, if an estrogen fluorescence intensity equal to five times the blank reading is acceptable, then 0.005  $\mu\text{g.}$  estrone can be estimated with ease and accuracy.

On the other hand, sulfuric acid fluorescence is not particularly specific for estrogens, and, in common with the other procedures mentioned, efficient preliminary separation of estrogens from interfering material is essential.

### 3. Enzymatic

Entirely different methods for the estimation of various steroid hormones, including estrogens, has been described by Talalay and Marcus (1956) and Hurlock and Talalay (1957, 1958). These methods depend on the interconversion of certain hydroxy- and ketosteroids by DPN (diphosphopyridine nucleotide)-linked hydroxysteroid dehydrogenases, obtained from the organism *Pseudomonas testosteroni*. Two hydroxy steroid dehydrogenases have been prepared in sufficient purity to be used in the estimation of urinary steroids (Hurlock and Talalay, 1958). The two enzymes are designated " $\beta$ " and " $\alpha$ ." The  $\beta$ -enzyme acts on hydroxyl groups in positions 3 $\beta$  and 17 $\beta$ , and can therefore be used to measure estrone, estradiol-17 $\beta$ , and estriol.

The enzymes have very high specificity for these particular groupings on the steroid molecule, and consequently this method of estrogen detection is more specific than the methods previously discussed. The enzymes can in fact be used to determine the purity of steroid samples. However, the enzyme cannot distinguish between the three classic estrogens, or between estrogens and other steroids having hydroxyl groups in the same positions. Consequently, standard extraction and chromatographic procedures still have to be employed (Hurlock and Talalay, 1958).

Currently, the sensitivity of the method is limited by the photometric determination of DPNH (dihydrodiphosphopyridine nucleotide), and lower limits of steroid which can be determined are approximately 0.3  $\mu\text{g.}$  This is not adequate for biological samples of low estrogen content, such as plasma. However, the use of fluorimetry for the determination of DPNH would probably overcome this objection (Lowry *et al.*, 1956). There is apparently no great interference from urinary chromogens in the photometric determination of DPNH, under the conditions described.

A sensitive technique for the estimation of estrone and estradiol-17 $\beta$ ,

using DPH-dependent isocitric dehydrogenase obtained from human placenta, has been suggested by Gordon and Villee (1956). A very high degree of specificity has been claimed by the authors.

## V. The Use of Isotopes

Although in the past, radioactive halogens have been incorporated into the estrogen molecule in order to study estrogen metabolism, the molecule was thereby considerably altered, and the results obtained could only be regarded as of limited value.

With the advent of readily available  $C^{14}$  and  $H^3$ , it proved practicable to synthesize these isotopes into the estrogen molecule, in place of inert atoms normally present. The resulting isotopically labeled estrogen could then be assumed to behave identically with the inert substance. Both estrone and estradiol-17 $\beta$  are available with either  $C^{14}$  or  $H^3$  incorporated into rings A or B which are relatively stable positions. Radioactive estriol is not currently available commercially.

Tritium-labeled estrogens have the advantage over their  $C^{14}$ -labeled counterparts in that the specific activity is much higher. Tritium-labeled estrone and estradiol-17 $\beta$  are available with specific activities up to 7.0  $\mu$ c. per microgram, compared with  $C^{14}$ -labeled estrogens of only 0.05  $\mu$ c. per microgram. With the appropriate instrumentation (windowless gas flow counter or scintillation counter), as little as 0.5 m $\mu$ c. of tritium can be detected. This confers a particular advantage on the use of the  $H^3$ -labeled steroids.

The use of radioactive estrogens has proved of the greatest value both in the estimation of inert estrogens and in the study of the metabolism of estrogens. However, certain precautions have to be observed. For instance, a high degree of radiochemical purity of the radioactive estrogen sample is essential. It is known that steroids of high specific activity undergo self-radiation decomposition, especially when stored in the dry state, and that estrogens, whether inert or radioactive, undergo decomposition in dilute solution in various solvents. Furthermore, it is entirely possible that an original radioactive estrogen sample may not be homogeneous. It cannot therefore be assumed that the requisite degree of radiochemical purity exists at any given time, although this is more likely to be so in recently synthesized material. Consequently, it is essential that the sample be subjected to a separation procedure (such as paper or partition chromatography) shortly before use to ascertain the extent to which the radioactive estrogen is in fact homogeneous, and to obtain a homogeneous sample.

Radioactive estrogens can be employed as follows in the chemical estimation of estrogens: Identification of curves following column partition chromatography; estimation of losses during hydrolysis, extraction, and other manipulations; isotope dilution; and reverse isotope dilution.

#### A. IDENTIFICATION OF CURVES FOLLOWING COLUMN PARTITION CHROMATOGRAPHY

If an inert (nonradioactive) estrogen and its radioactive isomer are chromatographed together on a partition column, a typical Gaussian curve is obtained which can be outlined both by radioactive measurement and by measurement of the inert estrogen. After appropriate adjustments of scale, the curves obtained by each method must superimpose. This method of outlining estrogen curves by two independent types of measurement is of considerable value in identifying these curves. For example, additional curves of unknown composition lying adjacent to estrogen curves are frequently met with in the chromatography of abnormal urinary extracts, and occasionally of normal urine extracts. If pure radioactive estrogens are added to the extract before chromatography, the superimposition of a "radioactive" curve on an "inert" curve will then provide important evidence of the identity of the inert curve, since adjacent interfering curves will not be so outlined. This procedure has been used by Preedy and Aitken (1961b). Radioactive estrogens can, of course, be similarly used with the other forms of chromatography.

The particular value of using tritiated estrogens is that no allowance need be made for the fluorescence of the (radioactive) estrogen added, since the small amounts used to give convenient count rates do not alter the fluorescence measurements significantly. The further use of isotopes in the identification of estrogens is discussed in Section VI.

#### B. ESTIMATION OF LOSSES DURING HYDROLYSIS, EXTRACTION, AND OTHER MANIPULATIONS

Since radioactive estrogens behave in the same way as the naturally occurring inert estrogens, radioactive estrogens may be added to an original sample of urine or plasma to determine losses during hydrolysis, extraction, and chromatography. If a known number of counts (as an authentic estrogen) are added, then the number of counts (as authentic estrogen) found after chromatographic separation divided by the number of counts originally added, will give the proportion of the estrogen lost during processing.

This procedure, however, has certain limitations. Except in rare in-

stances where only unconjugated estrogens are being determined, the addition of radioactive unconjugated estrogen before hydrolysis will not necessarily give an accurate estimate of any loss which occurs when inert conjugated estrogens are hydrolyzed. Although the addition of radioactive estrogens after hydrolysis would give an accurate estimation of losses occurring during further processing, it is doubtful whether their use is justified in every estrogen determination, particularly in normal urines or late pregnancy plasma samples, since the order of loss occurring during such determinations is already known (Brown, 1955; Preedy and Aitken, 1961b).

### C. ISOTOPE DILUTION

The technique of isotope dilution (and of reverse isotope dilution) has provided a valuable addition to steroid methodology, and has been successfully applied to estrogen determinations.

The method of estimating an inert estrogen by isotope dilution depends firstly on the addition of a known number of counts of an authentic radioactive estrogen to a sample containing the inert estrogen; and secondly, on establishing a ratio in a sample aliquot between concentration of the inert estrogen on the one hand and counts on the other. From this the amount of inert estrogen present in the original sample can be calculated by proportion. To establish such a ratio it is clear that neither the radioactivity measurement nor the inert estrogen estimation can be subject to significant interference. The estrogen (radioactive and inert) has to be separated efficiently from interfering material by extraction and chromatographic procedures as already described, or by some other procedure. Isotope dilution techniques cannot therefore be expected to provide any short cut to estrogen estimations.

In isotope dilution, the ratio between inert and radioactive estrogens is usually established either by countercurrent distribution or column partition chromatography. The superimposed radioactive and inert estrogen curves are drawn out, and the average ratio between the inert estrogen and counts in the "peak" tubes is taken. It is usually assumed that in the peak tubes (that is, the fractions at the top of the estrogen curves) radiochemical purity is adequate. But it is clear that for this to be true very efficient chromatographic separation must be obtained. This procedure has been used by Gallagher *et al.* (1958), and Preedy and Aitken (1961b). The value of isotope dilution lies in the fact that, since it depends on the ratio of inert to radioactive estrogen, extraction and transfer losses do not affect the estimation.

### D. REVERSE ISOTOPE DILUTION

In contrast to isotope dilution, reverse isotope dilution is designed to measure radioactive substances. Similar principles are involved. In a typical application, radioactive estrogen is administered to a human or an animal, and urine or plasma is collected for analysis of the radioactive metabolites. Hydrolysis and extraction procedures are carried out by methods already described, and at an appropriate stage a relatively large known amount of an authentic inert estrogen is added. The mixture then undergoes partition chromatography, and the estrogens are separated. The estrogen fraction then consists of a small amount of radioactive estrogen and a large amount of inert estrogen. Peak tube analysis is carried out as already described, or radiochemical purity established by repeated crystallization until the specific activity of the crystalline material remains constant. If repeated crystallization is to be carried out, about 50 mg. of inert estrogen is added. Peak tube analysis on the other hand requires the addition of only enough to ensure a convenient excess of estrogen in comparison with any interfering material present. In either case, a ratio between inert and radioactive estrogen is established. The amount of radioactive estrogen present in the original sample can be determined by proportion, since the amount of inert material added is known. Reverse isotope dilution has been used in the estimation of radioactive estrogens by Beer and Gallagher (1955a, b), Gallagher *et al.* (1958), and Levitz *et al.* (1956a, b), chiefly in studies of the metabolism of radioactive estrogens.

## VI. Further Identification Procedures

The procedures for separation and detection of estrogens so far described are in varying degree open to the objection that the identity of the estrogens has not been definitely established.

Although absolute identification of estrogens is difficult to achieve, it is possible to employ a series of additional identification procedures, so that the authenticity of an "estrogen" becomes progressively more firmly established. The physicochemical characteristics of the unknown substance are compared with those of a known estrogen standard, and if these are found to be the same by a varying number of tests, the unknown and the standard are for practical purposes considered identical. These

additional identification procedures have so far been used for particular applications only. Although many are time consuming or require special apparatus, it has become clear that at least some additional identification procedures should be used more frequently particularly in establishing the validity of new methods for estimating estrogens, and also in any new application of an established method.

There are a number of identification procedures which can be used, separately or (preferably) together. Several of the most important have already been discussed in the previous section on isotopes: superimposition of radioactive and inert estrogen curves obtained by partition chromatography, isotope dilution, reverse isotope dilution, and repeated crystallization to constant specific activity.

Other procedures which are used are: multiple chromatography; formation and chromatography of derivatives; infrared spectroscopy; bioassay of isolated material.

### 1. Multiple Chromatography

There are several ways in which multiple chromatography can be used, with or without the addition of radioactive estrogens. A single type of chromatography may be used (e.g., paper chromatography), with two different successive solvent systems. Crude material is chromatographed using one solvent system, separated material is eluted from an appropriate area of the paper and chromatographed again, using a second solvent system, comparison on each occasion being made with a standard.

Alternatively, two or more types of chromatography can be used. For example, after preliminary separation on an adsorption column, material from an appropriate fraction of eluent is chromatographed on paper (Meyer, 1955; Slaunwhite and Sandberg, 1956). Or, adsorption column chromatography may be followed by countercurrent separation (Levitz *et al.*, 1956a, b), or countercurrent separation may be followed by paper chromatography (West *et al.*, 1958a, b). In each case, comparison is made with an authentic estrogen standard.

A further procedure is particularly applicable to column partition chromatography. Crude material is chromatographed on one partition column. Fractions constituting the "estrogen" curves are collected and the chromatography repeated, using the same solvent system. By this means the major proportion of interfering material is removed before the second chromatography.

This method has been used by Preedy and Aitken (1961b) and Preedy *et al.* (1959). It is of great value in establishing the identity of estrogens in low-titer urine or in the other extracts, where the ratio of estrogen

to interfering material is particularly unfavorable. Extract from a relatively large volume of urine (e.g., 25% by volume of a 24-hour sample) is chromatographed in a large, or preparatory, partition column, capable of handling bulky extracts. The chromatographic position of the estrogen peaks is established by the fluorimetry of a small aliquot of each fraction. The appropriate fractions are then bulked and dried. Authentic tritiated estrogens are added, and the material chromatographed again on another partition column, using the same solvent system. Since most of the interfering material has been removed, conveniently large and well-separated estrogen curves are obtained, which can be outlined both by radioactive measurement and by sulfuric acid fluorescence. Although the same solvent system is used in both columns, the interfering material adjacent to each curve is first much reduced in amount, and is then redistributed in a Gaussian curve by the second chromatography, thus enabling the inert estrogen peak to be more clearly defined.

An example of the chromatography of a low-titer urine extract by routine single chromatography, and by double chromatography using radioactive estrogens, is given in Fig. 2.

## 2. Formation and Chromatography of Derivatives

The derivatives of estrogens most commonly used in identification of the parent steroid are monobenzoates (Gallagher *et al.*, 1958; Levitz *et al.*, 1956a, b), 3-methyl ethers (Brown, 1955; Diezfalussy and Linkvuist, 1956; Diezfalussy *et al.*, 1957; Engel *et al.*, 1958), acetates (West *et al.*, 1958a, b), thiosemicarbazone, and acetate and diacetate of 3-methyl ethers (Diezfalussy and Linkvuist, 1956). In practice, the untreated material is first chromatographed, and its chromatographic characteristics are compared with the appropriate estrogen. A derivative is then formed, and the material chromatographed again, using a different solvent system, comparisons being made with the authentic pure estrogen derivative. Either paper chromatography or partition chromatography can be used for the purpose, provided the system is of sufficient resolving power. When countercurrent separation is employed it is advisable to use at least 72 transfers. It is doubtful whether fewer transfers can reliably separate nonradioactive estrogen derivatives from interfering material with an approximately similar partition coefficient.

Isotopes are conveniently used in conjunction with the formation and chromatography of derivatives. The parent radioactive estrogen is added to an extract, which is then chromatographed, the radioactive and inert estrogen curves superimposing. A derivative is then formed and chromatographed. Superimposing radioactive and inert estrogen

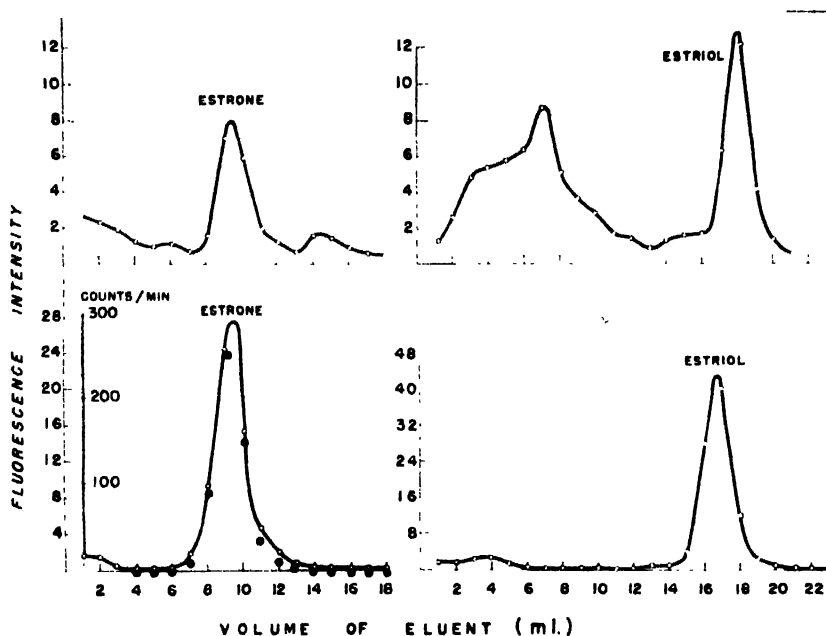


FIG. 2. Above, column partition chromatography of estrone and of estriol from a phenolic extract of female urine by the routine method of Preedy and Aitken (1961b). The volume of the eluate in 1-ml. fractions is plotted against fluorescence intensity. Below, the same urine extract subjected to double column partition chromatography as described in the text. It will be seen that virtually all interfering material has thereby been removed. Note the difference in the fluorescence intensity scale. In the lower estrone record, authentic tritiated estrone was added before chromatography. The estrone peak is outlined both fluorometrically (open circles) and by counts (black circles).

derivative curves are again obtained, the chromatographic characteristics of which correspond with those of the authentic derivative. Alternatively, when an inert derivative has been formed by appropriate treatment of an extract, the authentic radioactive derivative may then be added, and the mixture chromatographed. Inert and radioactive estrogen derivative curves will again superimpose. In the estimation of radioactive estrogens (as in metabolic experiments), a relatively large amount of authentic inert estrogen can be added to the extract and chromatographed in the usual way. Thereafter, a derivative can again be formed.

### 3. Infrared Spectroscopy

Infrared spectroscopy is the most recent addition to methods used for establishing the identity of steroids. In common with many other substances, estrogens absorb light in the infrared region. Absorption at some wavelengths is due to specific groupings on the molecule. In the



"fingerprint" region, the absorption pattern tends to be characteristic of each individual steroid. Consequently, this region is particularly useful for identification purposes. Infrared spectroscopy has not been used extensively in the identification of estrogens, mainly because only very small amounts of estrogen are usually available from natural sources. However, modern infrared spectrometers are available with attachments designed for very small samples, such as beam condensing units, and KBr discs in which the unknown or standard can be incorporated. It is claimed that under ideal circumstances, useful infrared spectra can be obtained with 5 to 10  $\mu\text{g.}$  samples. This appears to be so, from the experience of Oertel *et al.* (1959), using very small amounts of estrogen isolated from pregnancy plasma.

Where larger amounts of estrogen are available, such as in tissue analysis, estrogen infrared spectra have been more easily obtained (Hagopian and Levy, 1958; Diezfalusy and Magnusson, 1958). Under these circumstances, infrared spectroscopy constitutes a convenient and important method of identification.

Infrared spectrophotometers require a considerable degree of technical knowledge for their use and particularly for the interpretation of the spectra themselves. It should not be assumed that, because an infrared spectrum has been obtained and shows some similarity with that of pure standard, identity has been necessarily established. It is not advisable to use infrared spectroscopy as the sole criterion of identity.

#### 4. Bioassay of Isolated Material

Since the three classic estrogens, as well as at least some of the newly discovered estrogens can be bioassayed, this can be used as an important method of identifying estrogenic material obtained by chemical procedures. However, biological assay of estrogens can be extremely laborious, if maximal reliable information is to be obtained. About 20  $\mu\text{g.}$  each of estrone, estradiol-17 $\beta$ , and estriol must be obtained from the biological material in an adequate degree of purity. This is both difficult and time consuming if the material processed contains very little estrogen, as for instance, normal male urine. Since a statistically analyzable quantitative assay is required, and since estrogen assays are quantal, a 4-point design is used with 15-20 animals for each point, the activity of the unknown substance being directly compared with that of the appropriate estrogen standard. Under these conditions demonstration that an unknown estrogen has the same qualitative and quantitative biological activity as a known estrogen is very strong evidence of its identity. Such bioassays have been carried out for Preedy and Aitken (1961b) by Dr. John A. Loraine of Edinburgh on normal

male and female urines, as part of the procedures for validating the estrogen method of the former authors. In their studies, the material composing the actual chromatographic estrogen curves was subjected to bioassay.

Less elaborate bioassay procedures have been used for the same purpose by West *et al.* (1958a, b). It is probably even of some advantage to use "spot tests" of biological activity. Although of little quantitative value, these tests do at least show that the material under study is estrogenically active.

Bioassay clearly cannot conveniently be used to establish the identity of estrogens in multiple determinations, but it has particular value in providing important evidence for the specificity of chemical methods for estrogen estimation, or in providing additional evidence of the identity of estrogens isolated during a single study (West *et al.*, 1958a, b).

## VII. The Estimation of Conjugates

It has been known for many years that estrogens exist in the urine principally as conjugates (S. L. Cohen *et al.*, 1936), and it has been assumed therefore that estrogen conjugates also exist in the blood. This has recently been confirmed by the work of Sandberg and Slaunwhite (1957), Purdy *et al.* (1959), and Migeon *et al.* (1959). It appears that conjugation with sulfuric or glycuronic acid is an essential metabolic process, rendering the estrogens virtually inactive and relatively water soluble. The latter property may be necessary before renal excretion of estrogen metabolites can take place.

The direct estimation of estrogen conjugates is therefore of considerable interest, but until recently progress in this field has been minimal, due in great part to the absence of pure standard conjugates for testing the various procedures recommended. Methods used in the past have been in general somewhat crude and indirect and based on insufficient evidence regarding their validity.

However, progress has recently been made in finding more accurate methods for the estimation of conjugates. These, and some older methods, can be considered under the following headings: solvent partition; differential hydrolysis; chromatographic separation; and quantitative estimation.

### A. SOLVENT PARTITION

Preliminary partition between an aqueous phase and a high-polarity organic solvent such as diethyl ether (Diczfalusy and Linkvuist, 1956;

Diczfalusy and Magnusson, 1958), or chloroform (Migeon *et al.*, 1959) has been employed. In these partitions, it is assumed that "free" or unconjugated estrogens are extracted by the organic solvent, while conjugates remain in the aqueous phase. The former assumption is generally true, although it should be noted that Menini and Diczfalusy (1960) were unable to remove estriol quantitatively from the aqueous phase with chloroform. However, it is by no means certain that the conjugates remain quantitatively in the aqueous phase. For instance, W. H. Fishman and Sie (1956) were able to remove testosterone glucosiduronate from aqueous solution (pH 1.0) by ethyl acetate. Diczfalusy *et al.* (1957) did however obtain reasonably good recoveries of sodium estriol glucosiduronate added to various biological fluids using their procedure.

Alternatively, estrogen conjugates may be extracted from an aqueous phase by *n*-butanol (Schneider and Lewbart, 1959; Crépy *et al.*, 1957; Venning *et al.*, 1937; Dancis *et al.*, 1958). There is no general agreement on the optimal pH of the aqueous phase, but an acid pH has usually been employed. Butanol extracts of urine are invariably bulky. Information that this procedure is quantitative is again lacking.

### B. DIFFERENTIAL HYDROLYSIS

This has been partially discussed in Section IV, B. When conjugates have been separated from unconjugated steroids by one of the above solvent partitions, acid hydrolysis of the conjugates can then be carried out. The resulting "free" steroid can be measured, and from this an indirect estimate of conjugated steroid in the original sample obtained. This method has been used by Diczfalusy (1954) and Diczfalusy and Magnusson (1958).

The conjugates can be divided into sulfates and glucosiduronates by differential chemical or differential enzymatic hydrolysis. This was briefly discussed in Section IV, B. Certain chemical procedures appear to cause hydrolysis of sulfates only. These include treatment with 1,4-dioxane (Cohen and Oneson, 1953), or continuous extraction of an aqueous sample at pH 1.0 with ether (Burstein and Lieberman, 1958) or ethyl acetate. It appears from the work of the latter authors that continuous extraction is not necessary, hand extracting and allowing the solvent to stand for 24 hours at 38° being all that is required. Glucosiduronates are probably not affected by these procedures.

After hydrolysis and removal of the sulfates, the remaining aqueous material can be refluxed with strong acid as described in Section IV, B, the glucosiduronates (and presumably other conjugated material) being hydrolyzed by this method.

Enzymatic hydrolysis has been much used to differentiate glucosiduronates from sulfates. The  $\beta$ -glucuronidase preparation Ketodase and the phenolsulfatase preparation Mylase P, already referred to, are claimed to be free of sulfatase and  $\beta$ -glucuronidase contamination, respectively. An aqueous sample is incubated with one enzyme, followed by ether extraction of the free steroid, then incubation of the aqueous phase is repeated with the other enzyme, and a further ether extraction is carried out. Estimates of sulfates and glucosiduronates can then be obtained from analysis of the free steroid released by each form of hydrolysis. A combination of  $\beta$ -glucuronidase hydrolysis for glucosiduronates, followed by continuous ether extraction at pH 1.0 for sulfates, has been used by Slaunwhite and Sandberg (1956), West *et al.* (1958a, b), and Migeon *et al.* (1959).

These various procedures for estimating estrogen glucosiduronates and sulfates separately have yielded some useful information, but the methods are still somewhat crude. The differential procedures have not been fully tested out with pure conjugates. It is noteworthy that, after injection of radioactive estrogen into humans, and after both enzyme and acid hydrolysis of the urine, with extraction of the resulting free steroids, counts still remain in the aqueous phase, suggesting that even these procedures may not result in complete hydrolysis of the urinary estrogen conjugates (Beer and Gallagher, 1955a).

### C. CHROMATOGRAPHIC SEPARATION

Since the development of interest in the direct estimation of estrogen conjugates, attention has been given to their chromatographic separation, and a beginning has been made in devising suitable solvent systems.

Bush (1957) has pointed out that steroid conjugates are hardly more difficult to chromatograph than the parent steroids. The relative positions of the conjugates in chromatographic systems appear to be the same as in the case of the steroids themselves. This author has employed the solvent systems (or variants) given in Table VI in the chromatography of steroid conjugates in general either on paper or on Celite columns. These have proved useful in the separation of estrogen conjugates (Menini and Diczfalussy, 1960).

General observations on the fractionation and isolation of steroid conjugates have been made by Schneider and Lewbart (1959). Many of these observations apply to estrogen conjugates. Glucosiduronates behave usually as true weak acids, sulfates on the other hand tend to behave like weak bases, suggesting that partition systems using, respectively, aqueous alkaline or acid solutions as one phase may be useful. This was

TABLE VI

GENERAL PARTITION SYSTEMS FOR STEROID CONJUGATES\*

Stationary phase	Mobile phase
2 N $\text{NH}_4\text{OH}$	<i>n</i> -Butanol (or methyl ethyl ketone or ethylene dichloride)—3 parts, <i>tert</i> -butanol—1 part
30% (v/v) Aqueous acetic acid	Toluene—75-85 parts, <i>tert</i> -butanol—25-15 parts
30% (v/v) Aqueous acetic acid	Ethylene dichloride—75-90 parts, <i>tert</i> -butanol—25-10 parts

\* From Bush (1957).

found in general to be the case with adrenocorticoid conjugates, and in particular with androsterone and dehydroepiandrosterone conjugates. Solvent systems containing various mixtures of ethyl acetate, butanol, isopropyl ether, butyl ether, toluene, and hexane on the one hand, and 10% aqueous  $\text{NH}_4\text{OH}$  or 20% aqueous acetic acid on the other are recommended and can be used for paper or column partition chromatography. These observations indicate general lines along which further study of appropriate solvent systems for estrogens can be conducted.

Information on actual partition systems suitable for specific estrogen conjugates is sparse. Diczfalussy *et al.* (1957) used an ether: water system for the countercurrent distribution of sodium estriol glucosiduronate. Preedy *et al.* (1960) found that a system of 35% butanol-65% *n*-hexane (mobile phase), and 1% aqueous acetic acid (stationary phase) gave good separation of sodium estriol glucosiduronate using a Celite column (length 10 cm.). Partition coefficients and  $R_f$  values for estriol glucosiduronate in many solvent systems is given by Menini and Diczfalussy (1960). Estrone sulfate can be chromatographed on paper using 10% aqueous  $\text{NH}_4\text{OH}$  as the stationary phase, and *n*-butanol 50%-toluene 50% as the mobile phase (Levitz *et al.*, 1960).

One of the chief difficulties lies in the preparation of crude material for chromatography. Conjugates are usually extracted from an aqueous solution by *n*-butanol, but this procedure results in such a bulky extract when applied to urine, for instance, that subsequent direct paper or column partition chromatography is rendered impossible. Most efforts at preliminary purification of urinary conjugates have not proved successful. It is unfortunate that sample variations in the ion exchange resin Decolorite have made the use of this agent uncertain (Bush and Gale, 1957).

However, the use of absorption column chromatography seems promising, since these columns can frequently handle bulky extracts.

Crépy *et al.* (1957) applied butanol extracts of urine to alumina columns, and eluted the columns successively with butanol containing 2, 6, and 10% water and finally 15% 0.1 *N*  $\text{NH}_4\text{OH}$ . Sulfate esters were eluted by 6% water in butanol and glucosiduronates by 15% 0.1 *N*  $\text{NH}_4\text{OH}$  in butanol. The conjugates appeared to be adequately separated not only from one another, but also from much associated material. Such a column would appear to be a valuable preliminary step before the definitive chromatography of individual conjugates.

#### D. QUANTITATIVE ESTIMATION

Methods are available for the microdetermination of both sulfates and glucosiduronates. A method for estimating sulfate ions by rhodizonic acid has been applied to steroid sulfates by Schneider and Lewbart (1956). The naphtharesorcinol reaction has been used by Fishman and Green (1955) to measure steroid glucosiduronates. Both these methods are colorimetric. It is doubtful whether either are sufficiently sensitive for general use in the measurement of estrogen conjugates in biological fluids. The naphthoresorcinol method can measure down to 2  $\mu\text{g.}$  of glucosiduronate and the rhodizonic acid method down to 5  $\mu\text{g.}$  of steroid sulfate.

Fortunately, it seems that conjugation does not in general interfere with the usual methods for determination of the parent steroid (Schneider and Lewbart, 1959). Crépy *et al.* (1957) were able to apply a modified Kober reaction to measure estrogen conjugates, and Preedy *et al.* (1960) observed that both estradiol-17 $\beta$  and estriol glucosiduronates could be estimated directly by the usual sulfuric acid fluorescence procedure for the parent steroids (Preedy and Aitken, 1961b). This was also true of estrone sulfate. The great degree of sensitivity associated with sulfuric acid fluorescence is retained, the sensitivity comparing with that of the determination of the parent steroid. Presumably, the combined use of methods to detect both the parent steroid and the conjugating acid radical would have considerable value in the identification of specific steroid conjugates.

### VIII. The Estimation of Estrogens in Urine

There are two possible approaches to methods for estimating the three classic estrogens, estrone, estradiol-17 $\beta$ , and estriol, in urine and in other body fluids.

First, a method may be required for multiple or routine analyses.

Such a method should ideally combine simplicity with a high degree of sensitivity and specificity. Although procedures of adequate sensitivity are available, the simplicity of a method is usually inversely proportional to its specificity. It will be observed from the previous discussion that even an adequate degree of specificity is not easily achieved. Consequently, a compromise has to be reached, a method being chosen which is as specific as possible, but at the same time not unduly elaborate.

Second, a method may be required for the analysis of only a few samples, but with the object of virtually certain identification of the three estrogens. Under these circumstances, it is desirable and practical to use much more elaborate methods in order to establish the presence and identity of the estrogens without reasonable doubt.

Examples of both types of method will be discussed. It will be evident that the separate procedures embodied in the various methods will already have been described to some degree in previous sections.

#### A. METHODS FOR ROUTINE ANALYSIS

There are three principal complete methods for the estimation of urinary estrone, estradiol-17 $\beta$ , and estriol, which have been adequately tested and assessed. These are the methods of Brown (1955), Bauld (1956), and Preedy and Aitken (1961a, b).

The method for urinary estimation which is best known is that of Brown (1955). This method has the distinction of being the first published procedure sensitive and specific enough to measure estrone, estradiol-17 $\beta$ , and estriol in menstrual cycle urine. It involves acid hydrolysis, a double hand-extraction sequence (see Section IV, C), formation of the 3-methyl ethers of the three estrogens, followed by chromatography of these ethers in two alumina adsorption columns, and finally detection of the ethers by the Kober reaction, using the Allen spectrophotometric correction (see Section IV, F). The whole method has been extensively analyzed (Brown *et al.*, 1957a; Bulbrook *et al.*, 1957; Gallagher *et al.*, 1958), and much used (Diczfalussy *et al.*, 1957, 1959; Diczfalussy and Magnusson, 1958; Breuer *et al.*, 1957; Støa *et al.*, 1958; and others). Several authors have recommended modifications (e.g., Diczfalussy *et al.*, 1957). An additional purification step is now incorporated in the method (Brown *et al.*, 1957b).

As applied to urine, this method has certain disadvantages, in respect to specificity and sensitivity, which are inherent in the procedures it incorporates. Thus, the adsorption chromatographic system is not of great resolving power as compared with other systems (Section IV, E), and much interfering material may be included in the rather large

fractions of eluent collected and bulked. This creates particular difficulties in urines of low estrogen content. Furthermore, the alumina columns appear to be somewhat difficult to manage, a small variation in moisture content altering their performance considerably.

The Kober reaction measures much of the interfering material which is eluted from the alumina columns, and this has to be compensated for by the Allen correction. This correction relies on certain assumptions which may not be justified in every case, and, furthermore, the correction may be of a very high order compared with the "estrogen" reading (Ittrich, 1958). This may result in a falsely high estrogen value in certain circumstances (Diczfalusy *et al.*, 1958). In addition, the Kober reaction is much less sensitive than certain other methods for estrogen detection (Section IV, F).

The sensitivity of Brown's method is such that it can be used down to 5  $\mu$ g. of each estrogen per 24-hour urine sample. Although it has been used at levels below this, the loss of specificity is such as to throw considerable doubt in the validity of the method under these circumstances (Brown *et al.*, 1957a). Many normal male and female urines contain much less than 5  $\mu$ g. of each estrogen per 24 hours.

On the other hand, it is simpler and less laborious than the other established methods for the estimation of the three estrogens in urine, and is particularly well suited to the analysis of large numbers of specimens. Its use has contributed very considerably to our knowledge of estrogen metabolism in the last few years.

The method of Bauld (1956) involves acid hydrolysis, a double extraction procedure similar to that of Brown (1955), and chromatography on two Celite partition columns (one for estrone and estradiol-17 $\beta$ , one for estriol), followed by the Kober reaction, with the Allen correction. It differs significantly from the modified Brown method (Brown *et al.*, 1957a) only in the absence of methylation of the estrogens and in the type of chromatography used. The method has been used by the author (Bauld *et al.*, 1956) with success. It is satisfactory for measurement of each estrogen at levels higher than 3  $\mu$ g./24-hour urine (Bauld and Greenway, 1957).

The method of chromatography used by Bauld does not appear to yield the high resolution to be expected of column partition chromatography in general. (The procedure is described in detail by Bauld, 1955.) This is perhaps due to the calcining of the Celite supporting phase before use, the relatively large diameter (1 cm.) of the columns used, and the large volumes of eluent collected and bulked. These large fractions undoubtedly contain much interfering material, which is only partly removed by the next step of boiling with NaOH. The whole



method is rather more elaborate than that of Brown (1955). The disadvantages of the Kober reactions have already been mentioned.

A short method for the estimation of estriol only was published by Eberlein *et al.* (1958), which is in effect a simplified version of Bauld's method, involves enzymatic hydrolysis and adsorption column chromatography (alumina), followed by sulfuric acid fluorescence. Bauld and Greenway (1957) have also described a simplified method for the estimation of estrone.

The method of Preedy and Aitken (1961b) depends on acid (or enzyme) hydrolysis, a single hand-extraction sequence, partition column chromatography (using a single column), and detection of the estrogens directly by sulfuric acid fluorescence. This method has been much used by the authors over a period of years (Preedy and Aitken, 1955, 1957; Aitken *et al.*, 1958; Aitken and Preedy, 1958; Saffan *et al.*, 1960; C. H. Brown *et al.*, 1960).

The advantages of this method lie principally in the high resolving power and versatility of the particular chromatographic procedure employed. A single Celite column of about 0.5 cm. diameter is used, with 72% aqueous methanol as the stationary phase. Three successive mobile phases are employed, the changes being made in stepwise fashion by an automatic phase changer. A total of 75 to 80 ml. mobile phase is allowed to run through the column, and the eluate is collected in 1.0-ml fractions. The compositions of the successive mobile phases are as follows: first, 20% carbon tetrachloride-80% *n*-hexane; second, 15% carbon tetrachloride-11.2% chloroform-73.8% *n*-hexane; third, 48% chloroform-52% hexane (all per cents are v/v). The composition and volume of each mobile phase is designed so that estrone, estradiol-17 $\beta$ , and estriol are widely separated not only from one another but also from the large amounts of interfering material present in the urinary extract.

A diagram of the column, with an attached syphon calibrated to deliver 1.0 ml. and the automatic phase changer, is given in Fig. 3, together with a brief indication of the mode of action of the phase changer.

Each 1.0-ml. fraction is analyzed for estrogen content by fluorimetry, and the fluorescence intensity is plotted against the volume of eluent (or the fraction number). Such a chromatograph of a normal female urine is shown in Fig. 4. Although this method involves fluorimetry of 75 to 80 samples for each urinary estimation, the work can be reduced by suitable automation.

The high resolving power of this chromatographic system has certain great advantages as regards specificity. As described in Section IV, E,

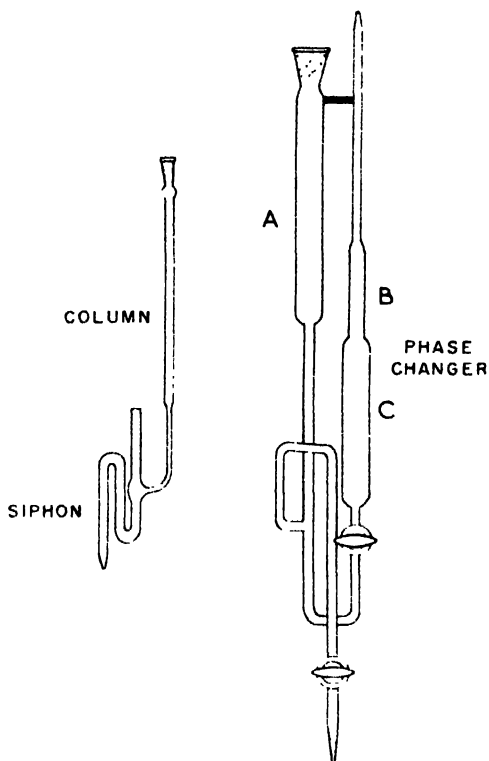


FIG. 3. Partition column, siphon, and automatic mobile phase changer used by Preedy and Aitken (1961b). The automatic mobile phase changer connects directly with the column. The phase changer is essentially a U-shaped reservoir, the two limbs of which contain two different solvents. The mixture of solvents delivered successively to the column by gravity depends on the relative internal diameters of the tubes A, B, and C.

in column partition chromatography single substances such as estrogens are distributed as Gaussian curves, when concentration is plotted against volume of eluent (Fig. 4). Small fractions of eluate are obtained so that each estrogen curve is composed of 4 to 6 points, and can therefore be drawn with reasonable accuracy. Individual estrogens can then be identified by (a) the presence of a curve in a position on the chromatogram corresponding with that occupied by the pure crystalline estrogen when similarly chromatographed, and (b) the presence of a Gaussian curve in this position. In this way, the identity of the estrogen curves can be monitored at each estimation.

This is of considerable value, since if these criteria are not met (e.g., in the case of an abnormal urine, or in urines of very low estrogen content), if there is doubt about the identity of a curve, or if an "estrogen" curve is found to be asymmetrical, then the situation is im-

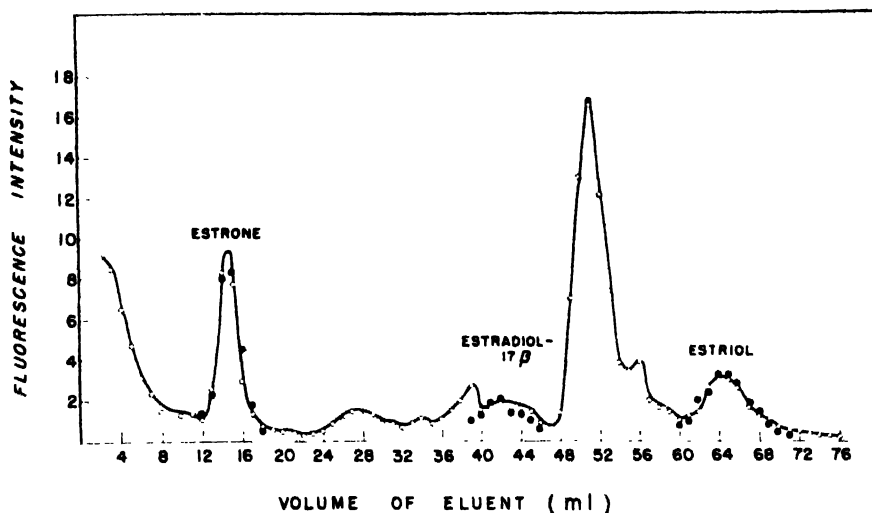


FIG. 4. Column partition chromatography of the phenolic extract of a normal female urine (11th day of the menstrual cycle). The volume of the eluate (in 1-ml. fractions) is plotted against fluorescence intensity. The estrone, estradiol- $17\beta$ , and estriol curves are labeled. Unlabeled curves are due to interfering material of unknown composition. Tracer amounts of authentic tritiated estrone, estradiol- $17\beta$ , and estriol were added to the extract before chromatography. The estrone and estriol curves are outlined by fluorimetry (open circles) and by counts (black circles), after appropriate adjustments of scale. Counts in fractions other than those composing the estrogen curves were negligible. The amount of (nonradioactive) estradiol- $17\beta$  present in this extract was below the minimum detection limits of the method ( $1.0 \mu\text{g. per 24-hour urine}$ ), no recognizable Gaussian curve being obtained by fluorimetry (Preedy and Aitken, 1961b). The amounts of estrogen in the urine sample were as follows ( $\mu\text{g. per 24-hour urine}$ ): estrone 3.3, estradiol- $17\beta < 1.0$ , estriol 3.7.

mediately apparent and further steps can then be taken to establish the identity of the estrogen more firmly. On the other hand, if large fractions of eluate are collected it will not be possible in the first place to construct an accurate chromatogram, no warning will be given of an abnormal situation, and much interfering material may be unknowingly measured as estrogen, resulting in an overestimate which may be considerable.

Methods for the further identification of estrogens have been described in Section VI. Those most conveniently employed after the routine column partition chromatography described above are repetition of the routine chromatography, after the addition of authentic tritiated estrogens, or the use of the double chromatography procedure with tritiated estrogens. This latter procedure is of particular value in those urines with low estrogen levels and large quantities of interfering ma-

terial. One or the other of these two procedures usually provides sufficient evidence of the identity of estrogens in urinary extracts. Further identification procedures (as described in Section VI) are in general required only for particular purposes.

The advantages of the method of Preedy and Aitken (1961a, b) are principally those of specificity, incorporated in a procedure which is nevertheless designed for the routine determination of urinary estrogens. In spite of the fact that each estrogen is divided between 4 to 6 fractions of eluate, sensitivity is actually higher than in the other two methods. The routine single chromatographic procedure can measure down to 1.0  $\mu\text{g}$ . estrone, 1.8  $\mu\text{g}$ . estradiol-17 $\beta$ , and 3.0  $\mu\text{g}$ . estriol per 24-hour sample, without significant loss of specificity, that is, with recognizable Gaussian curves on the chromatogram. With the double chromatographic system (Preedy *et al.* 1959), levels down to 0.2  $\mu\text{g}$ . of each estrogen per 24-hour urine, or even lower can be estimated with an actual gain in specificity over the routine procedure, as judged by the chromatographic curves obtained. (This system is described in Section VI.)

The disadvantages of the method of Preedy and Aitken (1961a, b) are that the chromatographic procedure takes 36 hours to complete, and 75-80 fluorescent determinations have to be carried out for each urinary sample. However, it is felt that the gain in specificity amply compensates for these disadvantages and it will be recalled from Section III that specificity is the most difficult of the criteria to achieve. The method therefore might be regarded as standing halfway between the simpler and less specific methods of Brown (1955) and of Bauld (1956) and the elaborate procedures designed to establish the identity of urinary estrogens mentioned next.

## B. METHODS DESIGNED PRIMARILY TO IDENTIFY URINARY ESTROGENS

Elaborate procedures have been used to establish clearly the identity of estrone, estradiol-17 $\beta$ , and estriol in urines, usually as part of metabolic experiments in which relatively few samples of urine were involved. These procedures require a great deal of work, and cannot therefore be used for routine urinary estimations. However, they are of the utmost value in providing definite evidence of pathways of estrogen metabolism, and have been employed to a great extent in studies involving the administration of radioactive estrogens to normal humans (Beer and Gallagher, 1955a, b; Sandberg and Slaunwhite, 1957; Migeon *et al.*, 1959) and in the study of estrogen metabolism in various disease states (West *et al.*, 1958a, b). Each study consists of an elaborate com-

bination of the procedures described in Sections IV, V, and VI, using various types of chromatography in sequence, formation and chromatography of derivatives, isotope and reverse isotope dilution, repeated crystallization to constant specific activity, biological assay of isolated estrogens, infrared analysis, etc.

The design of sequential procedures to establish the identity of estrogens without reasonable doubt depends on the particular application, and for details reference should be made to the various texts cited.

## IX. The Estimation of Estrogens in Blood, Tissues and Bile

### A. PLASMA

To date only two methods have been used extensively for the determination of plasma estrogen levels, the method of Preedy and Aitken (1961a, b) and a modification of the Brown (1955) method for urine, used by Diczfalusy and Linkvuist (1956), and Diczfalusy and Magnusson (1958).

The method of Preedy and Aitken (1961b) for plasma estrogens is similar to the method of these authors for urinary estrogens. It has also been used in metabolic studies by these authors for some years (Preedy and Aitken, 1957; Aitken *et al.*, 1958; Saffan *et al.*, 1960).

The method for urine has been fully discussed in the previous section, and many of the observations apply also to the procedure for plasma. The minor differences in processing are given in the published description (Preedy and Aitken, 1961b). In general, the method has been found quite satisfactory and of the requisite sensitivity for small samples (5-10 ml.) of fetal and maternal plasma estrogens in late pregnancy. There is much less interfering material than in urine, and consequently levels down to 0.05  $\mu$ g. of estrone and estradiol-17 $\beta$ , and down to 0.1  $\mu$ g. estriol/100 ml. plasma, can be estimated without loss of specificity. The method cannot be used for plasma estrogens determination in normal males or females, or before the second to third trimester of pregnancy, since in these circumstances estrogen levels appear to be below the minimum detection levels quoted above. Recoveries of estrogens added to plasma are similar to those for urine. The chromatogram from a normal late pregnancy plasma sample, before and after the addition of estrogens, is shown in Fig. 5.

A modification of Brown's urinary method used by Diczfalusy and Linkvuist (1956), and Diczfalusy and Magnusson (1958) appears satisfactory for the measurement of plasma estrogens, where relatively large volumes can be used. Because of the small amount of interfering mate-

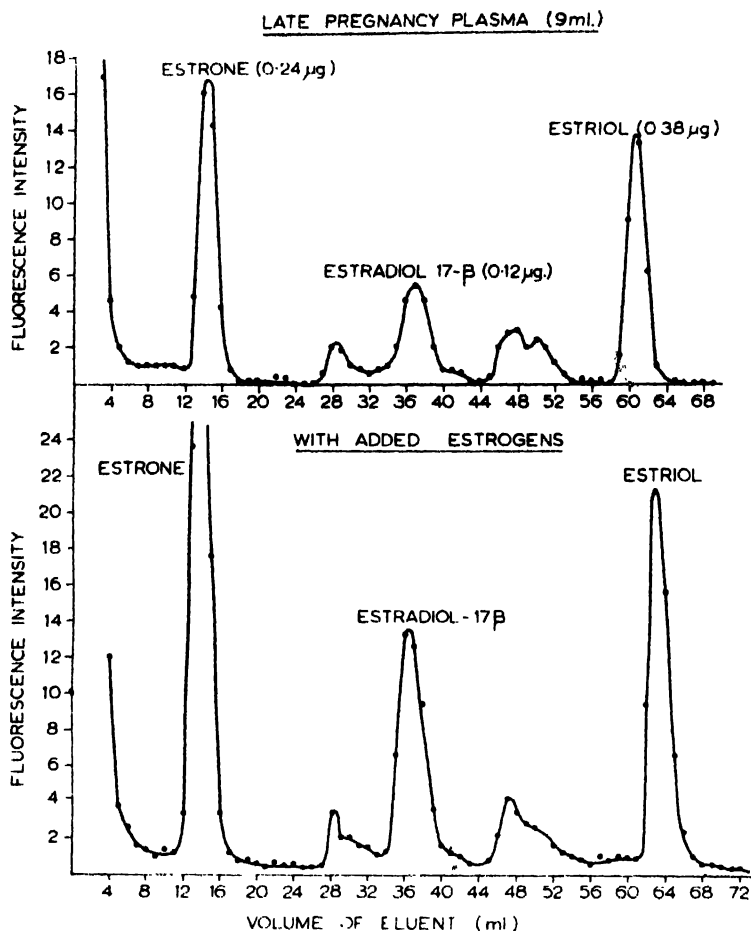


FIG. 5. Upper record, partition column chromatography of the phenolic extract of a late pregnancy plasma sample (Preedy and Aitken, 1961b). Curves due to estrone, estradiol-17 $\beta$ , and estriol are labeled, the amounts of estrogen represented by each curve being given in parentheses. Other curves are due to interfering material. Lower record, chromatography of an identical aliquot of the same plasma extract, to which crystalline estrone, estradiol-17 $\beta$ , and estriol had been added, showing a symmetrical increase in size of the three estrogen curves.

rial, large volumes of pooled plasma can be used to compensate for the lower sensitivity of the Kober colorimeter procedure. The method may not be of sufficient sensitivity to be used directly on the small plasma samples obtainable from a mother or a fetus (or infant) *in vivo*. However, in the hands of Diezfalussy and his co-workers, this method has yielded valuable information regarding fetal estrogen metabolism when used in combination with the further identification procedures described by them.

Elegant methods for studying radioactive estrogen metabolites in blood following the injection of estrone- $C^{14}$  and estradiol- $C^{14}$  have been devised by Sandberg and Slaunwhite (1957), Wall and Migeon (1959), and Migeon *et al.* (1959). The somewhat elaborate procedures used to establish the identity of the estrogens in their studies have been discussed in previous sections.

Isolation of estrone, estradiol-17 $\beta$ , and estriol from late pregnancy plasma has been carried out by Oertel *et al.* (1959), using two counter-current separations (160–200 transfers) and two paper chromatographies. These procedures were designed to obtain the three estrogens in sufficiently pure form to enable infrared spectroscopy to be carried out. They come under the same heading as similar elaborate procedures for urine, discussed in Section VIII, 2.

The difficulties posed by the presence of the plasma proteins in plasma estrogen determinations will be discussed at the end of this section.

## B. TISSUES, RED CELLS, BILE, AND OTHER MATERIAL

The concentrations of the various estrogens in tissues, red cells, bile, meconium, and at other sites, is of great importance in studying the metabolism of estrogens. In general, the methods used by various authors have already been discussed, either as complete methods, or in Section IV dealing with general procedures. Consequently, only specific points in methodology will be mentioned.

Estrogen concentrations in placental tissue have been determined by Mitchell and Davies (1954) and by Diezfalusy and Linkvuist (1956). In each case the placental tissue was ground up and extracted with large volumes of ethanol, the dried extract then being submitted to paper chromatography and sulfuric acid fluorescence in the first-mentioned study, and to adsorption chromatography and the Kober reaction in the second. Diezfalusy and Magnusson (1958) further estimated estrogens in fetal adrenals, kidney, and liver, using similar methods.

The concentration of estrogens in red cells has been examined by Wall and Migeon (1959), Migeon *et al.* (1959), and Sandberg and Slaunwhite (1957). In each case the cells were lysed and extracted with large amounts of ethanol. The extract was then submitted to various isolation procedures already discussed. Goldzieher *et al.* (1959) used Delsal's reagent (a mixture of methylal and methanol) for removing estrogens from red cell protein. Estrogens can only be removed quantitatively from protein with some difficulty, and Delsal's reagent appears to be the best solvent for this purpose.

Bile estrogens have been estimated by Sandberg and Slaunwhite

(1957) and by Adlercreutz, Diczfalusy, and Engstrom (1960). Estimations on meconium or fecal estrogens in the newborn have been carried out by Francis and Kinsella (1955), and by Diczfalusy *et al.* (1959). Estrogens in amniotic fluid and human semen have been studied by Diczfalusy and Magnusson (1958), and Diczfalusy (1954). In each case, the procedures used have already been discussed.

## X. The Problem of Protein Binding

The subject of protein binding of estrogens is of particular interest from the point of view of estrogen metabolism and transport, and several attempts have been made to study it. From a methodological point of view, there are two particular problems: the first, to devise adequate methods for studying the extent and nature of protein binding, and the second, to make sure protein binding is overcome when attempts are being made to estimate the total estrogen content of blood or tissues.

It seems virtually certain that some degree of protein binding of both estrogens and their conjugates occurs, particularly to plasma protein (Boettiger, 1946; Eik-Nes *et al.*, 1954; Slaunwhite and Sandberg, 1958). To what extent estrogens are bound to tissue proteins is not known, but a particularly tough protein-bound metabolite of estradiol-17 $\beta$  seems to be formed by rat liver homogenates (Riegel and Mueller, 1954).

Methods for studying protein binding of estrogens (as indeed of other steroids) are far from precise, and the extent to which these methods introduce artifacts into the experiments is unknown. As would be expected in these circumstances, results and conclusions tend to be variable. For instance, Diczfalusy and Magnusson (1958) found no apparent protein binding of estrogens in tissue or blood samples from the human fetus, in contradistinction to the findings of Diczfalusy (1953). The subject of the binding of steroid and steroid conjugates to human plasma protein has been reviewed by Sandberg *et al.* (1957).

The procedure most used for estimating bound and unbound estrogens has been the extraction of the protein-containing material with large volumes of ethanol or acetone. It is then assumed that the supernatant contains estrogens that were originally unbound to protein and the protein precipitate contains estrogens that were originally bound. However, there is every possibility that the addition of ethanol to the protein-containing fluid may significantly alter any protein binding that previously existed, either by releasing steroids from the protein bond or by binding steroid to the denatured protein.



When complete release of estrogens from protein binding is required, prior to estimation of total plasma estrogens, for instance, procedures that denature the protein as completely as possible seem to be the most effective. These include hydrolysis with strong acid under the conditions mentioned in Section IV, B (Preedy and Aitken, 1961b; Oertel *et al.*, 1959) or by digestion in hot 0.1 *N* NaOH (Sandberg *et al.*, 1957). These procedures certainly release some estrogens from the protein bond, but there is as yet no definite proof that either of these procedures are reliably quantitative. It appears that Delsal's reagent, which has already been mentioned, has advantages over ethanol or ether for extracting estrogens from the protein residues after such treatment (Goldzieher *et al.*, 1959).

## XI. The Estimation of the Newer Estrogens

The rapidly increasing number of newly discovered estrogens has already been referred to in Section I. Methods for estimating these estrogens (as opposed to isolating them) in various body fluids and tissues have in general not yet been worked out. For the methods of isolation, the reader is referred to the original reports mentioned in Section I.

Although many of the general procedures described in Section IV are applicable to the estimation of the newer estrogens, there are exceptions. Many of the newer estrogens are rendered unstable by boiling with hot acid, and this method of hydrolysis often cannot be used. Enzymatic hydrolysis is then the method of choice. Furthermore, many new estrogens are either partially insoluble (e.g., 2-methoxyestrone), or undergo rearrangement (e.g., 16 $\beta$ -hydroxestrone) in aqueous alkali. Consequently, the usual phenolic partition step, using NaOH and an organic solvent such as toluene, cannot be used in these circumstances. 16-Epi-estriol is a notable exception. It appears to be stable under both conditions.

Chromatography of various types has played a most important part in the isolation of the newer estrogens, and will presumably prove essential in methods for the routine estimation of these estrogens when such become available. A short summary of chromatographic procedures with appropriate solvent systems for these estrogens appear in Table VII. Other chromatographic systems are contained in the original reports of the isolation of these estrogens from natural sources.

Methods for the quantitative estimation of the newer estrogens are similar to those used for the three classic estrogens, estrone, estradiol-17 $\beta$ , and

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TABLE VII  
PAPER CHROMATOGRAPHY OF NEWER ESTROGENS

Estrogen	Solvent Systems <sup>a</sup>		<i>R<sub>f</sub></i> value	Reference
	Stationary phase	Mobile phase		
16-Epiestriol	Methanol 80% Water 20%	Isooctane 25% Toluene 75%	0.156	Migeon <i>et al.</i> (1959)
16-Oxoestradiol-17β	Methanol 80% Water 20%	Isooctane 25% Toluene 75%	0.311	Migeon <i>et al.</i> (1959)
16-Epiestriol	Methanol 70% Water 30%	Benzene 40% Petroleum ether 60% [Skelly C]	0.018	Migeon <i>et al.</i> (1959)
16-Oxoestradiol-17β	Methanol 70% Water 30%	Benzene 40% Petroleum ether 60% [Skelly C]	0.084	Migeon <i>et al.</i> (1959)
16-Epiestriol	Methanol 55% Water 45%	Benzene	0.438	Migeon <i>et al.</i> (1959)
16-Oxoestradiol-17β	Methanol 55% Water 45%	Benzene	0.640	Migeon <i>et al.</i> (1959)
16α-Hydroxyestrone	Formamide	Monochlorobenzene	0.05	Breuer and Nocke (1958)
16α-Hydroxyestrone	Formamide	Chloroform	0.41	Breuer and Nocke (1958)
16-Oxoestradiol-17β	Formamide	Chloroform	0.35	Breuer and Nocke (1958)
16-Epiestriol	Formamide	Chloroform	0.19	Breuer and Nocke (1958)

<sup>a</sup> For significance of percentages, see note to Table II.

estriol. It appears that all the newly discovered estrogens give rise to a Kober chromogen, although with some (e.g., 2-methoxyestrone) the chromogen may have an adsorption maximum different from that of the other estrogens. Sulfuric acid fluorescence may also be used. The fluorescence and absorption spectra of some of the newer estrogens in response to various wavelengths of exciting light has been studied by Bauld *et al.* (1960). Estrone, estradiol-17β, and the epimeric estriols develop fluorescence maxima in the region of 480 mμ; 16-oxoestradiol-17β, 16α-hydroxyestrone, and 16-oxoestrone at longer wavelengths (518–528 mμ), and 2-methoxyestrone at 579 mμ. Optimal conditions for obtaining sulfuric acid fluorescence with the newer estrogens have also been described by these authors.

## XII. Discussion

The application of newer physicochemical procedures to the separation of estrogens from interfering material has enabled complete methods for the determination of the small amounts of these hormones in biological fluids to be worked out.

Studies have in general proceeded along two lines. There are first, the simpler methods for routine use, designed principally for the determination of estrogens in urine and in plasma, and second, the more elaborate methods, designed to establish without reasonable doubt the presence and identity of various estrogens in small numbers of biological samples. Methods for both these purposes are now available, and their use has already added considerably to our knowledge of estrogen metabolism.

However, many problems in estrogen methodology remain to be solved. The most important are: the problem of interfering material, and therefore of specificity, when estrogens are measured in low-titer urine; the uncertainty of methods for studying the distribution of estrogens between conjugated and unconjugated forms; the lack of methods for the direct estimation of conjugates; the uncertainty of methods for studying the nature and extent of protein binding of conjugated as well as of unconjugated estrogens; the lack of methods for estimating many of the newer estrogens. The existence of these problems was referred to earlier in the chapter; they are common to the determination of many steroid hormones in biological fluids.

In spite of these difficulties, much valuable information regarding estrogen metabolism remains to be accumulated with the methods available at present. It is important, however, that the limitations of these methods should be clearly appreciated. An attempt to describe these methods and to define their limitations has been made in this chapter.

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## Chapter 2

# Assay of 17-Ketosteroids and Testosterone

RALPH I. DORFMAN

I. Introduction . . . . .	52
A. General Statement . . . . .	52
II. Determination of Dehydroepiandrosterone . . . . .	53
A. Urine Method of Burstein and Lieberman (1958) . . . . .	53
B. Plasma Method of Burstein and Lieberman (1961) . . . . .	54
C. Urine Method of Fotherby (1959) . . . . .	56
D. Urine Method of Munson <i>et al.</i> (1948) . . . . .	57
III. Determination of 5 $\alpha$ -Androst-16-en-3 $\alpha$ -ol (Brooksbank and Haslewood, 1960) . . . . .	59
IV. Determination of Testosterone in Plasma (Finkelstein <i>et al.</i> , 1961a) . . . . .	60
V. Determination of 17-Ketosteroids in Urine . . . . .	63
A. Methods of Dreker <i>et al.</i> (1947, 1952) . . . . .	64
B. Method of Hamburger and Rasch (1949) . . . . .	65
C. Method of Peterson and Pierce (1960) . . . . .	65
D. Method of Klendshoj <i>et al.</i> (1953) . . . . .	67
E. Method of Vestergaard (1951) . . . . .	67
F. Medical Research Council Method (1951) . . . . .	67
VI. Fractionation of Individual 17-Ketosteroids . . . . .	72
A. Method of Rubin <i>et al.</i> (1953b, 1954) . . . . .	73
B. Gradient Elution Method of Lakshmanan and Lieberman (1954) . . . . .	78
C. Gradient Elution Method of Kelle and Wade (1957) . . . . .	79
VII. 17-Ketosteroids in Plasma and Serum . . . . .	80
A. Method of Migeon and S'lager (1955) . . . . .	80
B. Method of Sauer <i>et al.</i> (1959) . . . . .	80
C. Method of Gardner (1953) . . . . .	83
VIII. The Zimmermann Reaction . . . . .	84
A. Method of Callow <i>et al.</i> (1938) . . . . .	84
B. Method of Holtorf and Koch (1940) . . . . .	84
IX. Preparation of Neutral Ketonic Fractions . . . . .	85
A. Method of Talbot <i>et al.</i> (1940) . . . . .	85
B. Method of Pincus and Pearlman (1941) . . . . .	86
X. Preparation of the $\beta$ -17-Ketosteroid Fraction . . . . .	86
A. Method of Frame (1944) . . . . .	87
B. Method of Butt <i>et al.</i> (1948) . . . . .	88
XI. Miscellaneous Methods . . . . .	88
References . . . . .	89

## I. Introduction

### A. GENERAL STATEMENT

The chemical determination of 17-ketosteroids in urine has been practiced extensively since Zimmermann (1935) reported that the  $\text{—CO—CH}_2\text{—}$  group of the steroid gives a reddish-purple color when reacted with *m*-dinitrobenzene in alkaline solution. The maximum absorption of the pigment is at 520  $m\mu$  when the ketone group involves carbon 17. Ketonic groups located at other positions in the steroid molecule give less intense pigment on a molar basis and there is a shift in the absorption maxima. The determination of 17-ketosteroids has been extended to plasma and considerable effort has been made to devise methods for the individual components. To evaluate the significance of 17-ketosteroid determinations, however, a consideration of the origin of these substances is required.

The principal urinary 17-ketosteroids are androsterone, etiocholanolone, dehydroepiandrosterone, 11-ketoandrosterone, 11-ketoetiocholanolone, 11 $\beta$ -hydroxyandrosterone, and 11 $\beta$ -hydroxyetiocholanolone, which comprise about 90% or more of the total 17-ketosteroids. These substances, with the exception of dehydroepiandrosterone, originate from various tissue precursors and the analysis of any single constituent represents no single precursor. Androsterone and etiocholanolone originate primarily from three  $C_{19}$  steroids, testosterone, androst-4-ene-3,17-dione, and dehydroepiandrosterone, and in the normal individual occur in roughly equal amounts. These two reduced steroids may also be formed from 17 $\alpha$ -hydroxyprogesterone and 11-deoxycortisol and these precursors form primarily etiocholanolone. The analysis of androsterone and etiocholanolone, no matter how reliably performed, cannot give a precise indication of any individual precursor. This situation also pertains to the 11-oxygenated 17-ketosteroids, since these four steroids arise from both cortisol and 11 $\beta$ -hydroxyandrost-4-ene-3,17-dione. The former steroid is converted primarily to the 11-oxygenated derivatives of etiocholanolone, while the latter compound is metabolized predominantly to the 11-oxygenated derivatives of androsterone. Dehydroepiandrosterone appears to indicate the endogenous production of this steroid and comes principally from the adrenal gland.

On the basis of the known interrelationship between the glandular steroids and the individual steroids, a method has been suggested to calculate the daily secretion of cortisol, 11 $\beta$ -hydroxyandrost-4-ene-3,17-dione, 17 $\alpha$ -hydroxyprogesterone, and 11-deoxycortisol, and the three  $C_{19}$  steroids dehydroepiandrosterone, androst-4-ene-3,17-dione, and testos-

terone (Dorfman, 1954). This type of information, even if the total 17-ketosteroid value is normal, may at times yield valuable specific information as to defects or abnormalities of individual constituents, but all too often severe clinical changes such as frank virilization in women may coexist with a normal total 17-ketosteroid titer and with a normal distribution of the individual constituents. This is possible because the *virilizing hormone* appears to be *testosterone*, and a small increase in daily production by an ovary, testis, or adrenal may produce profound biological effects without being reflected in significant changes in the 17-ketosteroids of urine or of blood. Thus the need for an analytical procedure for testosterone.

The need for knowledge of the blood testosterone titers is equally important in frank or marginal hypogonadism in men, since many investigators have observed eunuchoid or even castrate men who exhibit normal values of 17-ketosteroids. This chapter will present methods not only for the analysis of 17-ketosteroids of blood and urine, but a method for the assay of testosterone in plasma which appears to correlate reasonably well with the state of virilism of an individual.

## II. Determination of Dehydroepiandrosterone

### A. URINE METHOD OF BURSTEIN AND LIEBERMAN (1958)

Dehydroepiandrosterone is present in urine primarily as the sulfate and, for most analytical procedures, hydrolysis to the free compound is essential. The use of heat and acid resulted in great losses due to artifact formation (Dorfman and Shipley, 1956), and no single successful enzyme preparation is available to accomplish the necessary hydrolysis in a quantitative fashion. Two relatively simple procedures have been described by Burstein and Lieberman (1958) which eliminate the need for a lengthy continuous ether extraction procedure, as practiced earlier by Lieberman and Dobriner (1948).

The procedure consisted in making the urine 2 *N* with the proper amount of 50% aqueous  $\text{H}_2\text{SO}_4$  which is added cautiously with stirring. Ether is layered in an amount equal to that of the acidified urine and vigorously shaken in a cork-stoppered bottle 5 times daily for 4 days. This procedure hydrolyzed about 90% of the dehydroepiandrosterone.

Another procedure involves the solvolysis of urinary 17-ketosteroid sulfates in ethyl acetate. The urine is acidified to 2 *N* with  $\text{H}_2\text{SO}_4$  or acidified to pH 1 and brought to a salt concentration of 20% with NaCl. The sulfate conjugate is extracted quantitatively into ethyl acetate, and this solution is then kept at 38°C. for 24 hours, during which time a

quantitative hydrolysis of dehydroepiandrosterone and other sulfates (androsterone and epiandrosterone) is obtained.

Burstein and Lieberman (1958) described a model experiment in which potassium dehydroepiandrosterone sulfate equivalent to 10.6 mg. of the free steroid was dissolved in 50 ml. of a 20% NaCl solution and the mixture adjusted to pH 1 with  $\text{H}_2\text{SO}_4$ . An equal volume of ethyl acetate was used to extract the solution once and the separated organic phase kept at  $39^\circ\text{C}$ . for 3 hours, washed with sodium bicarbonate and water, and the ethyl acetate evaporated to dryness. Analysis of this residue indicated 10.8 mg. of free dehydroepiandrosterone. In a similar fashion 6.2 mg. of androsterone was recovered from an amount of potassium androsterone sulfate containing 6.8 mg. of free compound equivalent.

## B. PLASMA METHOD OF BURSTEIN AND LIEBERMAN (1961)

The solvolysis method described above for urine has been adapted to a plasma method for the estimation of both dehydroepiandrosterone sulfate and androsterone sulfate.

### 1. Collection of Sample

The procedure is applicable to not less than 10 ml. and not more than 30 ml. of plasma. The blood sample is treated either with heparin or is collected in a bottle containing dried potassium oxalate (1 ml. of 10% solution dried in an oven). As soon as the blood is cool enough to centrifuge, the plasma should be separated and then, if not used immediately, stored in a deep freeze. If a larger amount of blood is used the amount of oxalate must be increased.

### 2. Hydrolysis

The plasma sample is deproteinized with 3 times its volume of freshly distilled tetrahydrofuran (THF). THF is purified by refluxing for at least 2 hours with approximately 200 gm. per liter of NaOH or KOH flakes prior to distillation. One pours about one-half the volume of THF to be used for plasma sample into a 250-ml. round-bottomed flask, and transfers the plasma into the flask with the remainder of the THF. The flask is stoppered with a glass stopper and shaken vigorously. A weight of  $(\text{NH}_4)_2\text{SO}_4$  equivalent to 80% of the plasma volume is added, the mixture shaken vigorously and filtered through Whatman No. 1 paper in a Büchner funnel (100 mm. diameter). The filtrate should form two phases; if it does not, sufficient water is added so that the two phases do separate. The filtrate is transferred quantitatively into a 250-ml. separatory funnel, shaken well, and the lower phase discarded. The

upper phase is filtered through glass wool into a 250-ml. Erlenmeyer flask. The filtrate must be clear and free of water droplets. If not, filtration must be repeated. The contents of the flask are hydrolyzed by adding 0.09 ml. of 70% perchloric acid for each 100 ml. of THF. The flask is tightly covered with aluminum foil and placed in a 50°C. water bath for 2.5 hours. The hydrolyzate is transferred quantitatively with distilled THF into a 1-liter round-bottomed flask and neutralized with 1 ml. of concentrated  $\text{NH}_4\text{OH}$ . Evaporation *in vacuo* is carried to near dryness, thus removing practically all the organic solvent. The residual material is dissolved in ethyl acetate (100 ml.) and 10 ml. of 0.1 N NaOH is added, the flask stoppered, and shaken well. The contents of the flask are transferred quantitatively to a 250-ml. separatory funnel with ethyl acetate. After shaking, the lower phase is removed. If the plasma is icteric, a second alkali wash is done. The upper phase is washed to neutrality with three or four 20-ml. portions of distilled water. The emulsified interface is not removed until the last wash. The upper phase is transferred to a 1-liter round-bottomed flask and evaporated to dryness *in vacuo*. The residue is chromatographed on silica gel.

### 3. Silica Gel Chromatography

The adsorbent is standardized with dehydroepiandrosterone. It is recommended that the silica gel should be restandardized every 2 months. A slurry of 2 gm. silica gel, which is made with distilled methylene chloride, is poured into a chromatographic column (180 × 8 mm.). After settling, the adsorbent is washed with an additional 30–40 ml. portion of methylene chloride. The residue is dissolved to 3–5 ml. of methylene chloride and is quantitatively transferred to the column. The chromatogram is developed at a rate of 1 drop per second as follows:

Fraction No. 1	60 ml. $\text{CH}_2\text{Cl}_2$
No. 2	40 ml. of 0.5% $\text{CH}_2\text{Cl}_2/\text{MeOH}$
No. 3	20 ml. of 1% $\text{CH}_2\text{Cl}_2/\text{MeOH}$
No. 4	20 ml. of 2% $\text{CH}_2\text{Cl}_2/\text{MeOH}$
No. 5	20 ml. of 3% $\text{CH}_2\text{Cl}_2/\text{MeOH}$
No. 6	20 ml. of 10% $\text{CH}_2\text{Cl}_2/\text{MeOH}$

That fraction(s) is retained which the standardization procedure has shown to contain dehydroepiandrosterone. After removal of the solvent under nitrogen the residue is chromatographed on paper as shown in the following section. Fractions 3 and 4 usually contain the dehydroepiandrosterone.

### 4. Paper Chromatography

The residue obtained after silica gel chromatography is chromatographed on paper using the Bush A system which consists of 1 part

Skelly Solve B (mobile phase) and 1 part 90% methanol (stationary phase). Whatman paper No. 2 is used. With each run, standards of dehydroepiandrosterone and androsterone (40  $\mu$ g. of each per 2-cm. strip) are used. The residue is transferred to the starting line of the paper with methylene chloride and/or methanol. The paper is equilibrated in the tank overnight. After adding the mobile phase, the chromatogram is run for 5 to 6 hours. After the papers have been dried by hanging in air, the location of the standards on the chromatogram is determined by pulling the strip through 2.5 *N* KOH in absolute ethanol (stored in the freezer). The paper is blotted dry with filter paper and pulled through a solution of 2% *m*-dinitrobenzene in absolute alcohol. After blotting the strips dry, they are placed on a glass plate (56  $\times$  10 cm.) and heated in an oven at 60° until the color develops (2–4 minutes). Using the strip containing the standards as guide, the plasma strips are cut so that two areas are obtained. The dehydroepiandrosterone area (top) is cut 4 cm. above the corresponding standard and midway between the standard dehydroepiandrosterone and androsterone. The androsterone area extends from the mid-point to 4 cm. below the corresponding standard. Each strip is cut into small squares and allowed to stand overnight in 4 ml. of distilled methanol. Three milliliters of these solutions are evaporated to dryness in 15-ml. glass-stoppered centrifuge tubes and the residue used for the 17-ketosteroid determination as described in the following section.

#### 5. *Micro Zimmermann Determination*

To each dry residue add 0.1 ml. absolute ethanol and 0.1 ml. of 2% *m*-dinitrobenzene solution in absolute ethanol. Shake until the residue is completely dissolved. Add 0.1 ml. 5 *N* KOH in water (stored in the refrigerator). After mixing well keep the solution in the dark for 1 hour. Then add 0.3 ml. of distilled water followed by 1.5 ml. of freshly distilled THF. Stopper the tube and shake well. The upper phase is drawn off and filtered directly into 0.8-ml. microcuvettes through a 2-ml. Luer-Lok syringe fitted with a cotton-plugged needle and read in a Beckman DU spectrophotometer or equivalent at 440, 520, and 600  $m\mu$ . Dehydroepiandrosterone is used as the standard. The optical density readings obtained by the identical treatment of a corresponding strip of blank paper are subtracted from the observed values. The resulting estimates are further corrected by the Allen equation (page 159).

#### C. URINE METHOD OF FOTHERBY (1959)

Fotherby has described a relatively rapid and reliable method for the determination of urinary dehydroepiandrosterone, which in controlled

recovery studies indicated a recovery of 80–97% of added potassium dehydroepiandrosterone sulfate.

A 20-ml. aliquot of a 24-hour urine sample was placed in a flask fitted with a ground glass joint and heated, without adjustment of pH, under a condenser in a boiling water bath for 6 hours. The urine was cooled and extracted with 40 ml. of benzene and this in turn was washed with 20 ml. of water. Then 35 ml. of the washed benzene extract was measured into a boiling tube ( $6 \times 1$  in.) and evaporated to about 5 ml. at  $40^{\circ}$ – $60^{\circ}$  in a stream of air. The concentrated benzene solution was transferred to a  $6 \times 0.5$ -cm. alumina column prepared in benzene. Two milliliters of benzene was added to the tube and this solvent added to the column, followed by 8 ml. of benzene. The benzene eluates were discarded. Dehydroepiandrosterone together with  $6\beta$ -hydroxy-3,5-cycloandrostan-17-one were eluted with 30 ml. of 0.1% (v/v) ethanol in benzene and the eluates evaporated to dryness under a stream of air. The determination was completed using 20 and 40  $\mu$ g. of dehydroepiandrosterone standards and the Pettenkofer reaction (Munson *et al.*, 1948; Fotherby, 1958).

The method of Fotherby (1958) is essentially that of Munson *et al.* (1948) except that 0.2 ml. of acetic, 0.8 ml. of furfuraldehyde, and 3 ml. of  $H_2SO_4$  were employed

#### D. URINE METHOD OF MUNSON *et al.* (1948)

##### *Reagents*

1. *Furfural solution.* A commercial grade of furfural is distilled twice on a boiling water bath under reduced pressure. The middle fraction only is retained from each distillation. For a satisfactory product it is essential that the temperature of a boiling water bath not be exceeded. The nearly colorless product is promptly dissolved in 50% acetic acid at a concentration of 0.56% (v/v) and stored in the cold. The reagent, stored at  $-5^{\circ}$ , is stable for many months.

2. *Standard solutions of dehydroepiandrosterone (DIIA).* A solution containing 10 mg. of DIIA or 11.45 mg. of DIIA acetate/100 ml. of glacial acetic acid is prepared, and additional standards containing 0.060 and 0.020 mg. of DIIA per milliliter are obtained by dilution with glacial acetic acid. Because DHA crystallizes in two polymorphic modifications, the melting point is an unsatisfactory criterion of purity. For the greater portion of the work the acetate of this compound [m.p.  $169^{\circ}$ – $170.5^{\circ}$  (corrected);  $[\alpha]_D = +4.2^{\circ}$  (ethanol)] was used as the standard, but the results were expressed in terms of DHA. The acetate gives the same intensity of color mole for mole as the free hydroxyketone.

3. *Sulfuric acid, 16.0 N.*

4. *Acetic acid, 50% by weight.*

Provided that all test solutions are treated uniformly, minor changes in reagent concentrations, temperature, and time are relatively unimportant.

An amount of pure steroid or of urine extract estimated to contain 10–50  $\mu$ g. of DHA or its equivalent is transferred to a calibrated Evelyn colorimeter tube and evaporated to dryness on a water bath under a stream of nitrogen. The dry residue, if the evaporation has been properly carried out, is confined to a small area in the bottom of the tube and is dissolved in 0.5 ml. of glacial acetic acid, with warming if necessary. The solutions in Table I are then added to Evelyn tubes (or equivalent) in duplicate or triplicate and mixed.

TABLE I  
REAGENTS FOR DEHYDROEPIANDROSTERONE (DHA) DETERMINATION

	Glacial acetic acid (ml.)	DHA standard solution (ml.)	50% Acetic acid (ml.)	Furfural solution (ml.)	Urine extract in glacial acetic acid (ml.)
Blank without furfural	0.5	.	2.0		
Reagent blank	0.5			2.0	
DHA standards (10-, 30-, and 50- $\mu$ g. levels)		0.5		2.0	
Urine extract blank			2.0		0.5
Urine extract				2.0	0.5

To each tube indicated in Table I, 7.5 ml. of 16 *N* sulfuric acid are added at 1-minute intervals. After the contents are mixed well, the tube is placed in an efficient, large-capacity, constant-temperature water bath maintained at  $67^{\circ} \pm 0.2^{\circ}$ . After exactly 12 minutes in the bath, the tube is removed and immediately placed in an ice bath for 1 minute. After all the tubes have been heated and cooled (a series of 30 to 40 tubes can be analyzed conveniently), the color intensity is determined in the Evelyn colorimeter (or equivalent), with Filter 660, the wavelength of maximum absorption of the colored product. A center setting is obtained by adjusting the "blank without furfural" to 100. Since all the solutions containing furfural increase slightly in color while standing at room temperature, the colorimetric measurements are made at approximately



the same time interval ( $\pm 10$  minutes) after removal from the bath.

There is a small but significant day-to-day variation in the color intensity developed by DHA standards, even though the same reagents are used and the assay conditions are apparently identical. It is therefore essential that a full set of standards be included in each assay series. The color produced is affected significantly by changes in furfural concentration,  $\text{H}_2\text{SO}_4$  concentration, bath temperature, and heating time. Therefore it is also essential to use the same reagents and assay conditions for standards, blanks, and extracts.

#### *Calculations*

1. The galvanometer readings ( $G$ ) are converted to  $L$  values ( $L = 2 - \log G$ ). (A convenient table for conversion is included in the manual accompanying the Evelyn colorimeter.) If replicate determinations have been made, the mean  $L$  value for each set of replicates is calculated.

2. To eliminate nonspecific color ( $a$ ) originally present in the extract and ( $b$ ) produced by the action of  $\text{H}_2\text{SO}_4$  alone on the extract, the  $L$  value for the "urine extract blank" is subtracted from that of the "urine extract." Occasional urine extracts develop a slight turbidity, which also is corrected by the urine extract blank. The mean  $L$  values of the reagent blank and the DHA standards are plotted on graph paper, and a curve is drawn connecting the points. (The curve deviates slightly from strict linearity, thus differing from that obtained in the analysis of cholic acid.) The DHA content of the urine extract aliquot is estimated by interpolation on the graph, and the total DHA content of the extract is obtained by application of the appropriate factor.

### **III. Determination of 5 $\alpha$ -Androst-16-en-3 $\alpha$ -ol (Brooksbank and Haslewood, 1960)**

The inclusion of the determination of this steroid in this chapter is based on the evidence that  $\Delta^{16}$  compounds are metabolites of testosterone, and that the urinary concentration of the  $\Delta^{16}$  compound may in fact be a measure of the daily testosterone production. Increased urinary concentration of 5 $\alpha$ -androst-16-en-3 $\alpha$ -ol is associated with hirsutism and virilism in women, and men's urine has a higher concentration than that of women's urine. Cholesterol and pregnenolone are biosynthetic precursors of this  $\Delta^{16}$  steroid (Burststein and Dorfman, 1960). Incubation of 4- $\text{C}^{14}$ -testosterone with rat testis and human liver homogenates have yielded  $\Delta^{16}$  compound (Stylianou *et al.*, 1961a, b).

A preliminary method for the estimation of the 5 $\alpha$ -androst-16-en-3 $\alpha$ -ol in urine has been suggested (Brooksbank and Haslewood, 1960) which

TABLE II

EXCRETION OF URINARY 5 $\alpha$ -ANDROST-16-EN-3 $\alpha$ -OL:  
PRELIMINARY RESULTS ON THE URINE OF HEALTHY INDIVIDUALS<sup>a</sup>

Subject	Sex	Approximate age	Milligrams androstenol/24 hours
1	M	32	<0.2, 0.22, 0.26, <0.2, <0.2
2	M	38	2.13, 2.05
3	M	28	0.93
4	M	24	1.55
5	F (pregnant)	35	<0.2
6	F	23	0.70
7	F	70	<0.2
	M (pooled urine)	<45	1.06 mg./per liter

<sup>a</sup> From Brooksbank and Haslewood (1960).

is based on the color tests devised by Miescher (1946) for the detection of 17 $\alpha$ -hydroxy- and  $\Delta^{16}$ -C<sub>19</sub> steroids. Here 5 $\alpha$ -androst-16-en-3 $\alpha$ -ol is dissolved in acetic acid containing 0.5 gm./100 ml. (w/v) resorcyaldehyde and heated at 100° with an equal volume of 5% (v/v) sulfuric acid in acetic acid. The resulting blue color absorbs maximally at 585 m $\mu$  (initially also at 540 m $\mu$ , according to conditions). With purified reagents, the blank is negligible and the agreement with Beer's law, if a colorimeter with a green filter is used, is excellent; quantities down to 2-3  $\mu$ g. in 4 ml. of solution can be readily measured. The color reaction is markedly specific.

Brooksbank and Haslewood (1960) prepared a suitable extract for the colorimetric determination of 5 $\alpha$ -androst-16-en-3 $\alpha$ -ol by incubating urine buffered to pH 4.5 with a  $\beta$ -glucuronidase preparation from limpets and extraction of the liberated steroids with an organic solvent, preferably ether. The extracted material was separated on a simple alumina column with petroleum ether-benzene (1:1 v/v). Some preliminary results are presented in Table II. The low excretion figures for subject 1 were not due to poor recovery, since 74-85% of the steroid glucuronoside added to the urine was recovered. No evidence for the presence of the sulfate conjugate could be found.

#### IV. Determination of Testosterone in Plasma (Finkelstein *et al.*, 1961a)

In a previous section (page 53) the need for a method for the determination of testosterone in blood was discussed. It is pertinent to

mention that this androgen has been detected in human urine by Schubert (1960), who claimed that unspecified human urine contains about 50  $\mu\text{g}$ . of testosterone per day's urine. If this finding is substantiated, it is possible that urinary determinations of this steroid would be practical and meaningful.

The method of Finkelstein *et al.* (1961a) for measuring testosterone in blood is based on the fact that testosterone can be enzymatically converted to estradiol-17 $\beta$  and estrone (Ryan, 1959) and these latter compounds can be estimated in extremely minute quantities by a fluorometric technique (Finkelstein *et al.*, 1947; Finkelstein, 1952). Testosterone in concentrations of 0.1  $\mu\text{g}$ /100 ml. of human plasma may be estimated.

The following method has been developed:

1. Plasma was extracted 4 times with equal volumes of ether:chloroform (3:1) and the combined extracts were washed with 1 *N* NaOH to remove acidic and phenolic compounds and finally washed with saturated  $\text{NaHCO}_3$  and water. The neutral extracts were dried over  $\text{Na}_2\text{SO}_4$  and concentrated to dryness.

2. The dried extract, containing the neutral compounds, was dissolved in 70% methanol, stored in a deep freeze overnight, and when a precipitate formed this was rapidly centrifuged and the clear supernatant partitioned against petroleum ether.

3. The methanol was removed by distillation under reduced pressure and the resulting aqueous residue extracted with equal volumes of benzene 3 times. The combined benzene extracts were washed once with water, dried over  $\text{Na}_2\text{SO}_4$ , and concentrated to dryness. The dried residue was chromatographed in the ligroin-propylene glycol system (Savard, 1953) in parallel with a testosterone standard for 72 hours at 27°C.

4. The testosterone zone was located by viewing in the short ultraviolet. The material contained in this testosterone zone was incubated with a placental enzyme preparation in the presence of 0.5  $\mu\text{M}$  TPN, 5  $\mu\text{M}$  glucose-6-phosphate, 0.5 Kornberg units glucose-6-phosphate dehydrogenase, 50  $\mu\text{M}$  phosphate buffer at pH 7.2 and 0.154 *M* KCl to make a final volume of 1.0 ml. The incubations were carried out for 1.5 hours at 37°C. in air in a Dubnoff metabolic shaker.

The enzyme was prepared according to Ryan (1959). Each pellet obtained after 105,000 *g* centrifugation was resuspended in 3.0 ml. of phosphate buffer (0.1 *M*, pH 7.2) and 0.5 ml. of the suspension was used as the source of enzyme. The activity of the enzyme preparation was checked with each run by incubation of 4-C<sup>14</sup>-testosterone and determining the extent of conversion to estradiol-17 $\beta$  by both counts and fluorom-

etry. The placental preparation was found to be stable after several months' storage in the deep freeze.

5. The reaction was stopped by adding 10 volumes of ethanol. Insoluble material was removed by centrifugation and the precipitate washed with one-half the original volume of ethanol. The ethanol supernatants were combined and evaporated to dryness. The dried residues were moistened with a little ethanol and then taken up in 10 ml. benzene. The benzene solution was extracted once with 10 ml. and twice with 5 ml. 1 *N* NaOH and the pooled alkali extracts were placed in an ice bath and the pH adjusted to about 8.0 with concentrated HCl; extraction was with a 3 × 10-ml. portion of benzene. The benzene extracts were washed successively with 3.0 ml. 30% H<sub>2</sub>SO<sub>4</sub>, water, saturated NaHCO<sub>3</sub>, and again with water to neutrality. The benzene extracts were dried over Na<sub>2</sub>SO<sub>4</sub> and then evaporated to dryness in a stream of nitrogen. The dried residues were chromatographed on paper in the benzene:methanol system along with standard estradiol-17β and estrone. The respective zones were eluted and quantitatively estimated fluorometrically (Finkelstein, 1952; Finkelstein *et al.*, 1961b). In model experiments, 4-C<sup>14</sup>-testosterone was added to plasma, converted to estrogenic material enzymatically, and the conversion products were localized on paper by scanning. The concentration was estimated both by counting and by fluorometry.

6. To control the over-all recovery and to assist in detecting the zones on paper, 0.01 μg. or less of C<sup>14</sup>-testosterone or H<sup>3</sup>-testosterone with a high specific activity may be added to the 50-ml. sample of plasma.

*Recovery experiments.* Three-tenths microgram of 4-C<sup>14</sup>-testosterone (9000 c.p.m.) was added to 50 ml. of plasma and subjected to the procedure outlined above. In four experiments (Table III) the major conversion product was estradiol-17β and the over-all mean recovery was

TABLE III  
RECOVERY OF ADDED 4-C<sup>14</sup>-TESTOSTERONE FROM HUMAN PLASMA<sup>a</sup>

Plasma sample	Per cent recovery as estradiol-17β after chromatography based on:	
	c.p.m.	Fluorometry
I	55	63
II	47	58
III	55	58
IV	44	50

<sup>a</sup> From Finkelstein *et al.* (1961a).

50% (as estradiol-17 $\beta$ ) on the basis of the C<sup>14</sup> count, and 55% when the product was measured fluorometrically.

*Estimation of testosterone in normal plasma.* Five plasma samples, including three from normal men and two from normal women, were analyzed for testosterone content. The values in two men were 0.2 and 0.4  $\mu\text{g.}/100$  ml. of plasma and the value was less than 0.1  $\mu\text{g.}$  in a third male subject. In the two women, plasma values of 0.1 and less than 0.1  $\mu\text{g.}/100$  ml. of plasma were found (Table IV).

TABLE IV  
TESTOSTERONE IN HUMAN PLASMA<sup>a</sup>

Subject	Sex	Age <sup>b</sup>	Testosterone ( $\mu\text{g.}/100$ ml plasma)
1. Normal	M	40	<0.1
2. Normal	M	35	0.2
3. Normal	M	28	0.4
4. Normal	F	35	0.1
5. Normal	F	22	<0.1
6. Adrenal adenoma (F.M.)	F	27	1.3
7. Ovarian hilus-cell tumor (H.H.)	F	71	2.0

\* From Finkelstein *et al.* (1961a).

<sup>b</sup> Because of the limited number of samples, no significance should be attached at present to the relationship of age to testosterone levels in plasma.

Plasma samples from two female patients with virilizing syndromes were also studied. The patient F.M. was suffering from an adrenal adenoma and excreted a gram or more of 17-ketosteroids daily. The other patient (H.H.) had a hilus-cell tumor of the ovary with exceedingly low urinary 17-ketosteroids. In patient F.M., 1.3  $\mu\text{g.}/100$  ml. plasma and in patient H.H., 2.0  $\mu\text{g.}/100$  ml. plasma were found.

## V. Determination of 17-Ketosteroids in Urine

This group of steroids specifically include androsterone, dehydroepiandrosterone, etiocholanolone, 11-ketoandrosterone, 11-ketotiocholanolone, 11 $\beta$ -hydroxyandrosterone, and 11 $\beta$ -hydroxyetiocholanolone as the principal components and all share the property of a ketone group at position 17. The determination may be performed at different levels of complexity. Methods are available and will be discussed in this chapter which give an accurate index of the total of all the 17-ketosteroids, while

other methods provide for the quantitative analyses of the individual components.

Certain micromethods will be described which have the advantage of dealing with small volumes of urine and are suitable for clinical studies in which quantitation of the individual components is not required.

#### A. METHODS OF DREKTER *et al.* (1947, 1952)

*Method A (1947).* Ten milliliters of urine and 3 ml. of concentrated HCl are placed in a 125-ml. Erlenmeyer flask, and the flask is stoppered with a Pyrex flat-headed stopper. The flask is heated in a water bath at 80°C. for 10 minutes, and 5 ml. of the hydrolyzate are cooled and transferred to a 125-ml. separatory funnel. Twenty milliliters of ether are added, and the funnel is shaken for 30 seconds. The extracted urine is removed. The ether is washed once with 10 ml. of 10% NaOH and once with 10 ml. of distilled water, and shaken for 10 seconds with each wash. Five milliliters of ether are removed, evaporated, and assayed by means of the Zimmermann reaction [see Drechter method B (1952)].

*Method B (1952).* Ten milliliters of urine are transferred to a 30-ml. Pyrex centrifuge bottle. Three milliliters of concentrated HCl are added, and the bottle containing the mixture is placed in a water bath at 100°C. (With each batch of determinations a method blank is set up by substituting 10 ml. of water for urine and following through with the entire procedure.) The bottle is removed from the water bath after 10 minutes and cooled. Ten milliliters of ethylene dichloride are added, and the bottle is stoppered and shaken for 15 minutes. The bottle is centrifuged, and the top aqueous layer is aspirated. The ethylene dichloride extract is poured through Whatman's No. 1 filter paper into a 20-ml. bottle. Approximately 20 pellets of sodium hydroxide are added, and the bottle is stoppered and shaken for 5 minutes. The solution is filtered through Whatman's No. 1 filter paper. Two milliliters of the ethylene dichloride solution are transferred into a test tube and dried in a water bath at 100°C., until all traces of solvent have been evaporated. When cool, 0.4 ml. of 1% *m*-dinitrobenzene in absolute ethanol is added, and the tube is rotated to dissolve the dried material. Then 0.3 ml. of 8 *N* potassium hydroxide is added, the contents are mixed and placed in a water bath at 25°C., after which 0.4 ml. of the standard (5 mg. of dehydroepiandrosterone in 100 ml. of absolute ethanol) is transferred to a test tube, and the *m*-dinitrobenzene solution is added as above. Similarly, a Zimmermann reagent blank is prepared. After exactly 25 minutes, 2 ml. of 75% ethanol are added. The Zimmermann reagent blank is set at 100% transmission. The standard is read. Next, the method blank is

set at 100% transmission. The unknown is now read. The standard is equivalent to 10 mg. per liter of 17-ketosteroids expressed as dehydro-epiandrosterone, and the calculation of the unknowns is based upon the recommendation of the manufacturer of the colorimeter employed. If the colorimeter to be used has a capacity greater than 2.7 ml., then a larger aliquot of the ethylene dichloride extract can be taken, and the amounts of reagents used for the Zimmermann test are increased as needed.

### B. METHOD OF HAMBURGER AND RASCH (1949)

An amount of urine equal to one-fiftieth of a 24-hour output is measured out from a 10-ml. pipet graduated to 0.1 ml. into a flask (if below 10 ml., water is added to 10 ml.). From a 1-ml. pipet graduated to 0.01 ml., 10 volume % of 40%  $\text{H}_2\text{SO}_4$  are added to the urine.

*Simultaneous hydrolysis and benzene extraction.* Forty milliliters of benzene (crystallizable) are added to the acidified urine. The boiling is performed on an electric hot plate in 450-ml. flat-bottomed culture flasks fitted with reflux condensers. An electric hot plate with a diameter of 22 cm. leaves space for 3 of the flasks, and the reflux condensers can be connected serially. When the temperature on the hot plate is kept moderate, the urine boils smoothly, especially when a piece of glass is put into the flask. The advantage of a flask of this shape is the extended contact surface between the urine and the benzene layer; with 20 ml. of urine the contact surface is about 80 sq. cm., whereas the height of the urine layer is merely  $\frac{1}{4}$  cm. The liberated steroids, therefore, pass very rapidly to the benzene layer. Refluxing with benzene for various periods of time showed that most of the steroids are removed within 5 minutes. Since the hydrolysis usually is complete after 25 minutes, the total time for the benzene refluxing should be 30 minutes.

After being refluxed for 30 minutes, the mixture is cooled under running tap water, and the benzene extract is freed from nonspecific chromogenic substances and phenols by extracting once with saturated  $\text{NaHCO}_3$  solution, twice with 2 *N*  $\text{NaOH}$  solution, and twice with water, each of the washings being made with about 10 ml. of solution. After being dried with anhydrous sodium sulfate, the benzene extract is filtered and evaporated to dryness over a boiling water bath under reduced pressure. The residue may now be analyzed by the Zimmermann reaction.

### C. METHOD OF PETERSON AND PIERCE (1960)

A 5-ml. aliquot of urine is placed in a 40-ml. graduated ground-glass-stoppered conical centrifuge tube containing 0.5 ml. of concentrated  $\text{HCl}$

(C.P.). The tube is covered with a marble and heated in a boiling water bath for 20 minutes. After cooling, 25 ml. of a 1:1 mixture of petroleum ether-benzene is added and the tube shaken for 15 to 20 seconds. By means of a capillary pipet the aqueous layer is removed and the remaining solvent layer is washed successively once with 1/15 volume of 5% KOH and twice with 1/10 volume of water. A 20-ml. aliquot of the washed solvent containing the total neutral 17-ketosteroids is transferred to a ground-glass-stoppered tube and evaporated to dryness in a water bath (40°–45°C.) under a stream of air. At this temperature it is not necessary to use nitrogen at this step.

The dried residue is dissolved in 0.4 ml. of ethanolic *m*-dinitrobenzene. Reagent blanks in duplicate are prepared containing 0.4 ml. of the ethanolic *m*-dinitrobenzene solution. Standards are prepared in duplicate containing 30  $\mu$ g. of dehydroepiandrosterone plus 0.4 ml. of ethanolic *m*-dinitrobenzene. Then 0.2 ml. of 5.0 *N* KOH is added to each tube and incubated at 25°  $\pm$  2°C. in the dark for 90 minutes. After incubation 3.0 ml. of 50% ethanol is added, mixed, 3.0 ml. of dichloromethane added, stoppered, and the mixture shaken vigorously for 10 seconds. After standing in the dark for about 5 minutes, when two layers separate, 3 ml. of the dichloromethane layer are removed, transferred to a cuvette containing a few drops of ethanol, and the optical density measured against a water blank at 520  $m\mu$ .

#### Calculations

$$\frac{DU}{DS} \times 0.03 \times \frac{TVU}{UA} = \text{Milligrams of 17-ketosteroids per day}$$

DU = Optical density of unknown

DS = Optical density of standard

0.03 = Milligrams of standard (dehydroepiandrosterone)

TVU = Total 24-hour urine volume

UA = Urine aliquot used, milliliters

(20-ml. aliquot solvent equivalent to 4 ml. of urine)

The method of Peterson and Pierce (1960) is particularly valuable because estimation of the true total 17-ketosteroids is possible without the need for a Girard separation, column chromatography, or the use of a color correction equation. This is so because the dichloromethane extraction procedure removes the pink color (see also page 85), absorbing at 520  $m\mu$  from the undesirable brown pigments which absorb at 420  $m\mu$ . When this method is used, 10–24 mg. per day of 17-ketosteroids are



found for normal men's urine and normal women's urine varies from 6 to 14 mg. per day.

#### D. METHOD OF KLENDSHOF *et al.* (1953)

This is essentially the methods of Drekter *et al.* (1947, 1952) except for the following modifications; methyl Cellosolve was used as a diluent, the *m*-dinitrobenzene was dissolved in this solvent, 4 *N* KOH was used in place of 8 *N* aqueous KOH, and the color developing reaction was run for 60 minutes at room temperature. A more stable color was obtained and in replicate analysis excellent agreement was found. Steroid recovery varied from 96 to 104%.

#### E. METHOD OF VESTERGAARD (1951)

(For Survey of Procedure, see page 68)

The correction for nonspecific chromogens was made according to the method of Gibson and Evans (1937). The nonketonic fraction of a pooled urine was made using the Girard reagent (Pincus and Pearlman, 1941) and the ratio between the extinction at 420 and 520  $m\mu$  determined to be 2.0, while that of a pure sample of dehydroepiandrosterone was 0.33. The correction formula was as follows:

$$E_{520 \text{ corr}} = \frac{2.0 E_{520} - E_{420}}{167}$$

The method gave a coefficient of variation of 3.4 and is simple to perform. The author records the fact that a single operator could perform between 60 to 72 analyses in an 8-hour working day.

#### F. MEDICAL RESEARCH COUNCIL METHOD (1951)

The Medical Research Council (Great Britain) Committee on Clinical Endocrinology has published a standard method for the determination of 17-ketosteroids in urine. This method is reproduced here with the permission of this group.

##### 1. Collection of Urine Specimen

Since there is considerable diurnal variation in excretion of 17-ketosteroids (Pincus, 1943), complete 24-hour collections of urine are necessary. Subjects should be instructed to reject the first morning urine on the first day of collection and then to collect all urine up to and including the first morning urine on the second day. Addition of preservatives is

## SURVEY OF THE PROCEDURE

Pipet off 0.3 ml. of conc. HCl in specially made glass-stoppered centrifuge tubes

Add 2 ml. of urine

Place on boiling water bath for 17 minutes

Cool

Add 4 ml. of ethyl ether with a syringe pipet

Extract in shaking apparatus for 1 minute

Suck away urine

either

Add 4 ml. of distilled water

Suck away

Add 15-20 NaOH pellets

Shake (100 times in shaking apparatus)

or

Add 2 ml. of 2 N NaOH

Shake and suck away

Add 4 ml. of distilled water

Suck away

Add 2 ml. of distilled water

Shake and suck away

Dry by adding one spatula of  $\text{Na}_2\text{SO}_4$

Shake

Filter through sintered-glass filters into test tubes

Washings with two 1-ml. portions of ether

Evaporate on water bath at  $50^\circ\text{C}$ .

To each test tube add 0.24 ml. of a freshly made solution consisting of 2 parts 1.25 N alcoholic KOH solution and 1 part 2% *m*-dinitrobenzene solution

Dissolve the residue by thorough shaking of test tube

After 60 minutes in the dark at  $25^\circ\text{C}$ . add 3.76 ml. of absolute alcohol read in Beckman colorimeter against blanks

Compare with standards

Apply correction formula

usually unnecessary. Formation of inconvenient amounts of ammonium carbonate can be prevented by addition of a salt of a heavy metal (to inhibit urease activity), e.g., copper sulfate 1 mg. per milliliter.

### 2. *Hydrolysis and Extraction of Urine*

The following method, based on the work of Robbie and Gibson (1943), has been found to be convenient and rapid. Direct comparison with other recommended procedures has shown close agreement in amounts of 17-ketosteroid extracted.

A sample of urine (100 ml.) is brought to boiling under reflux over a Bunsen burner flame. Concentrated HCl (10 ml.) is added down the condenser and boiling maintained for 10 minutes. The urine is then allowed to cool somewhat and 30 ml. of carbon tetrachloride added down the condenser. The contents of the flask are again maintained at the boil for 10 minutes. The flask is then cooled, the CCl<sub>4</sub> layer removed and replaced by the same volume of fresh solvent, and the mixture again refluxed for 10 minutes. The CCl<sub>4</sub> layer is then removed and added to the first extract.

The total CCl<sub>4</sub> extract (about 60 ml.) is washed successively with: (1) 20 ml. water, (2) 20 ml. 2 *N* NaOH, (3) 20 ml. water, (4) 20 ml. water containing a pinch of sodium dithionite (Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>). The washed CCl<sub>4</sub> extract is then evaporated to dryness on a water bath using a water-pump vacuum to remove last traces.

The dry residue is dissolved in aldehyde-free absolute ethanol. The volume of ethanol may be varied according to the expected ketosteroid content. With normal urines 4 ml. of ethanol is convenient, but 2 ml. or even 1 ml. may be more appropriate for urines of low ketosteroid content. Ethanolic extracts thus prepared appear to be quite stable and need protection only from evaporation.

### 3. *Colorimetric Estimation*

a. *Ethanol*. The suitability of the absolute alcohol is the most important factor in achieving satisfactory results. Some grades of commercial "absolute" alcohol can be used without preliminary purification but usually some treatment is necessary. The following method described by Callow *et al.* (1938) may be employed.

Commercial "absolute" alcohol is treated with 4 gm. per liter of *m*-phenylenediamine hydrochloride, allowed to stand in the dark for a week, with occasional shaking, and then distilled, the head and tail fractions being rejected.

This purified alcohol should be used for all purposes in connection with the estimation of 17-ketosteroids.

*b. m-Dinitrobenzene.* A well-crystallized and fairly pure specimen is further purified thus: 20 gm. is dissolved in 750 ml. of 95% ethanol, warmed to 40°C., and 100 ml. of 2 *N* NaOH is added. After 5 minutes, the solution is cooled and 2500 ml. of water is added. The precipitated *m*-dinitrobenzene is collected on a Büchner funnel and washed very thoroughly with water, sucked dry, and recrystallized twice in succession from 120 and 80 ml. of absolute ethanol. The material should be well crystallized in almost colorless needles, m.p. 90.5°–91°C.

The reagent is a 2% w/v solution of this material in absolute ethanol. It is stored in a glass-stoppered brown bottle and kept in the dark. Under these conditions it is stable for 10–14 days.

*c. Potassium Hydroxide.* The reagent solution is 2.5 *N* KOH in absolute ethanol; KOH (9 gm.) is dissolved with shaking or mechanical stirring in 50 ml. of absolute ethanol and the solution filtered through a hardened filter paper. The concentration is checked by titration with acid (methyl orange indicator) and adjusted between the limits 2.48 to 2.52 *N*. The solution is stored in a refrigerator and must be discarded as soon as the faintest color is perceptible.

Wilson and Carter (1947) have reported that the 2.5 *N* potassium hydroxide alcoholic solution can be kept for a period of 3 to 4 months if ascorbic acid is added, and if the reagent is kept in an atmosphere of nitrogen. This modified reagent is prepared as follows:

One hundred milliliters of absolute alcohol are measured into a glass-stoppered Pyrex bottle and chilled in crushed ice. Two lots of 15 mg. each of ascorbic acid are weighed. The first lot is ground up with small successive portions of absolute alcohol and added to the chilled alcohol. Since ascorbic acid is sparingly soluble in ethanol, it is added as a fine suspension. This must be done before dissolution of the solid potassium hydroxide and again after filtering the resultant cloudy solution. Next 25 to 30 gm. of finely powdered potassium hydroxide pellets are added. A stream of nitrogen is bubbled gently through the mixture for 5 seconds, the stopper replaced under nitrogen, and the bottle shaken vigorously for about 5 minutes. It is necessary to shake until a concentration of at least 2.6 *N* is reached. After the mixture is sampled to test the normality, the air space is again flushed with nitrogen if further shaking is required. The solution is filtered through a fine porosity Büchner-type Pyrex glass-fitted filter into a 250-ml. Pyrex suction flask packed in crushed ice. A gentle stream of nitrogen is played over the surface of the filtering fluid from a conical funnel. The second lot of 15 mg. of ascorbic acid is ground with absolute alcohol and added to the filtered solution. The final concentration of the cold alkali is then adjusted to 2.5 *N* within 1%.

A slight turbidity from the ascorbic acid may be disregarded. The reagent is stored in the cold under nitrogen. Thereafter the air space is always flushed with nitrogen after the bottle has been opened.

The following tubes should be always set up:

- (1) Reagent blank—0.2 ml. ethanol, 0.2 ml. *m*-dinitrobenzene reagent (DNB), 0.2 ml. KOH.
- (2) Urine extract—0.2 ml. urine extract, 0.2 ml. DNB, 0.2 ml. KOH.
- (3) Standard—0.2 ml. standard, 0.2 ml. DNB, 0.2 ml. KOH.

A convenient standard is one containing 0.1 mg. androsterone or dehydroepiandrosterone in 0.2 ml. absolute ethanol.

The tubes are stoppered and placed in a thermostat at  $25^{\circ} \pm 1^{\circ}\text{C}$ . for 60 minutes. During incubation the tubes should be protected from bright light. It is most convenient to keep them in complete darkness.

At the end of 60 minutes 10 ml. of absolute ethanol is added to each tube and the contents mixed. The urine extract and the standard are then read in a photoelectric colorimeter against the reagent blank tube.

Colorimeter readings should always be made with two different filters, one green (approximate wavelength maximum 5200 Å), and one blue or violet (approximate wavelength maximum 4300 Å). These correspond to "Ilford Spectrum" filters Nos. 604 and 601, respectively. Readings with both filters are necessary for correcting for interfering chromogens by the method given below.

There is a straight-line relation between extinction and amount of ketosteroid up to about 0.1 mg. of androsterone. With larger quantities of androsterone the calibration deviates from a straight line. If very high readings are obtained it is necessary to dilute the original urine extract and repeat the color development. Dilution of the final colored solution should not be employed, as it gives erroneous results.

#### 4. Correction for Interfering Chromogens

Substances other than 17-ketosteroids develop color with the reagents. Talbot *et al.* (1942) suggested the use of the following formula for correcting the observed green extinction values for interfering chromogens:

$$\text{Corrected green} = \frac{\text{Observed G} - 0.6 \text{ V}}{0.73}$$

That is, from the observed green extinction subtract 6/10 of the observed violet extinction and divide by 0.73. The corrected green extinction is converted into milligrams of standard by comparison with the extinction of the latter with the green filter. The validity of this correction has been checked by comparison with values obtained on urinary neutral ketone

fractions obtained by the use of Girard's reagent (Talbot *et al.*, 1942) and by simultaneous determinations by colorimetric and polarographic methods (Butt *et al.*, 1951).

Kenny (1960) has published detailed directions for the construction of a nomogram for the computation of the color value for 17-ketosteroid estimations by the Zimmermann reaction.

## VI. Fractionation of Individual 17-Ketosteroids

Callow and Callow (1940) separated androsterone, dehydroepiandrosterone, and etiocholanolone by a chromatographic method, using aluminum oxide as the adsorbent and mixtures of carbon tetrachloride and ethanol for the development and elution. This method was sufficient for semiquantitative work and for qualitative identification of this limited group of 17-ketosteroids. With the discovery that additional 17-ketosteroids were present in human urine, it was desirable to have a method for the quantitative determination of the individual constituents. Dingemans *et al.* (1946) published such a method, employing alumina as the adsorbent and benzene and benzene-ethanol mixtures for elutions. 17-Ketosteroid determinations were done on the individual eluates. The individual components were identified by the solvent fraction in which they were found, as compared to reference steroids, and by isolation of crystalline material from some fractions. This technique was used with modification by Robinson and Goulden (1949), Devis (1951), Pond (1951), Zygmuntowicz *et al.* (1951), and Rubin *et al.* (1953a). These methods were not satisfactory because of the inherent difficulties in obtaining standardized adsorbents and sharp separations of individual components. A further difficulty was due to the fact that the procedures involved exposing such compounds as 11 $\beta$ -hydroxyandrosterone and 11 $\beta$ -hydroxyetiocholanolone to heat and acid, which dehydrated these steroids to their  $\Delta^9(11)$ -dehydro derivatives. The  $\Delta^9(11)$  steroids were not separable from their corresponding reduced steroids, androsterone, and etiocholanolone by the adsorption chromatographic procedures employed.

The introduction of a paper chromatographic method for the separation of the individual 17-ketosteroids by Rubin *et al.* (1953b, 1954) proved to be a considerable advance, since good resolution was possible, and a method was introduced that permitted the quantitation of 11 $\beta$ -hydroxyandrosterone and 11 $\beta$ -hydroxyetiocholanolone. This method will be described in detail. The use of gradient elution methods developed for 17-ketosteroids especially by Lakshmanan and Lieberman (1954) and Kellie and Wade (1957) proved to be important advances and are herein

described. Definitive, essentially symmetrical peaks of the individual components can be obtained with these procedures.

### A. METHOD OF RUBIN *et al.* (1953b, 1954)

#### 1. Preparation of Chromatograms

Whatman No. 1 filter paper is cut into strips 14 or 16 cm. wide and 56 cm. long. These strips are washed in a Soxhlet apparatus with 1:1 methanol-benzene for approximately 48 hours. For quantitative paper chromatograms these strips are cut as shown in Figs. 1 and 2. From each

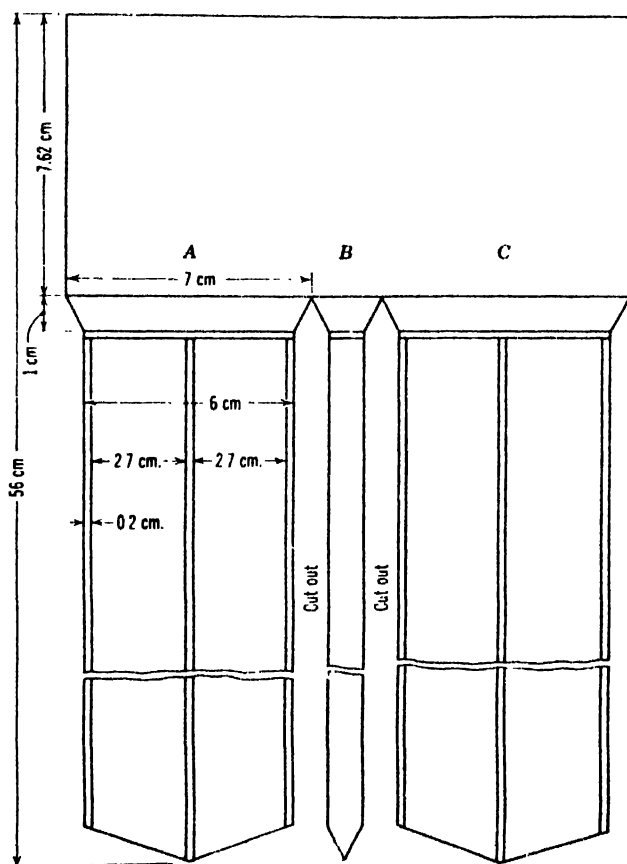


FIG. 1. Preparation of filter paper for quantitative determination of individual 17-ketosteroids (Rubin *et al.*, 1953b, 1954).

16-cm. strip, two quantitative strips of 6 cm. each and one qualitative strip of 1 cm. (for crystalline standards) are obtained. The 6-cm. strips are ruled vertically 2 mm. from each edge, and a 2-mm. strip is marked down the center. These 2-mm. strips are used for locating the zones of

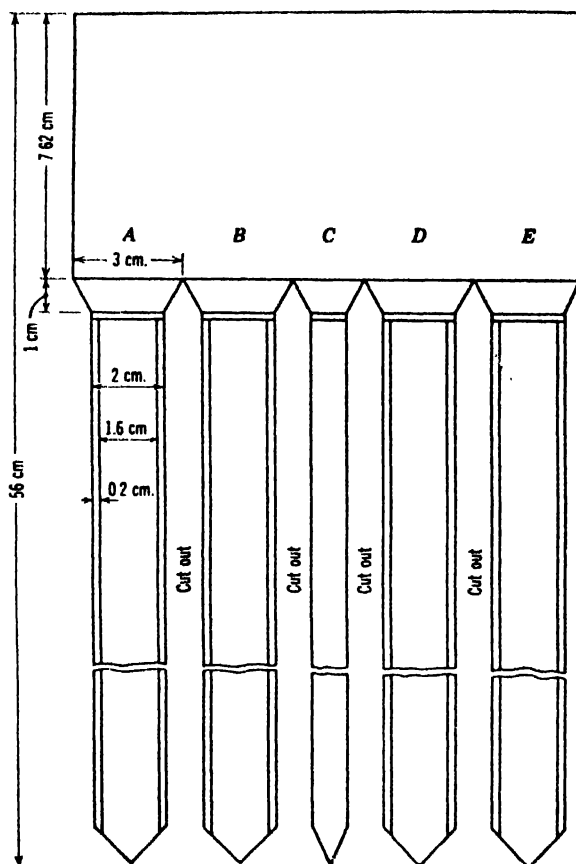


FIG. 2. Preparation of filter paper for quantitative determination of individual 17-ketosteroids (Rubin *et al.*, 1953b, 1954).

Zimmermann-reacting material and stapled back into their original positions in the chromatogram, all being mounted on a sheet of paper. The remaining areas of these zones, 5.4 cm. in width and corresponding in length to the Zimmermann-reacting zone on the 2-mm. strips, are used for elution and quantitative determinations. The 14-cm. sheets yield four 2-cm. strips and one 1-cm. strip. The 2-cm. strips are also ruled off 2 mm. from each edge. The area remaining for quantitative determination was 1.6 cm. in width.

Just before the urine extracts or crystalline steroids are put on the paper, the paper is impregnated with a 1:1 mixture of either propylene glycol and methanol or phenyl Cellosolve and methanol. The excess is removed by blotting. For reproducible results, these two mixtures should be freshly prepared.

The solutions for quantitative determination are made up in benzene



and applied at the starting line as evenly as possible across the width of the strip, using micropipets (usually 100  $\mu$ l.), and drying the solution on the paper with a stream of nitrogen. Ten-microliter aliquots of each solution are removed for quantitative Zimmermann determinations (page 69). The standard reference compounds are placed on the 1-cm. strip, the exact amounts not being determined. A minimum of 5  $\mu$ g. was used for the reference standard to ensure visualization with the Zimmermann reagent.

Descending chromatograms were run with, as the mobile phase, heptane previously equilibrated with either phenyl Cellosolve or propylene glycol. The tanks are sealed with starch-glycerol paste (Burton *et al.*, 1950). It is essential to run the heptane-phenyl Cellosolve chromatograms in a tank maintained at  $23^{\circ} \pm 2^{\circ}\text{C}$ . The heptane-propylene glycol chromatograms give satisfactory resolution at temperatures ranging from  $23^{\circ}$  to  $42^{\circ}$ . The times of development will be discussed in detail in connection with chromatography of the urine extracts. The propylene glycol chromatograms are allowed to dry by hanging them in the air at room temperature, but the phenyl Cellosolve chromatograms require drying in an oven at  $90^{\circ}\text{C}$ . for approximately 2 hours (until all odor of phenyl Cellosolve has disappeared), as small traces of phenyl Cellosolve interfere with the Zimmermann reaction.

17-Ketosteroid-containing zones from the 6-cm. strips are eluted quantitatively by allowing them to stand overnight in 95% ethanol and washing them repeatedly. The ethanol is removed *in vacuo*, and all samples are made up to 10 ml. in benzene before aliquots are taken for analysis. The zones from the 2-cm. strips are not eluted quantitatively. They are placed overnight in test tubes containing 10 ml. of 95% ethanol, and aliquots are taken directly from this solution. These steroids are recovered in amounts ranging from 85 to 117% after application of amounts as low as 90  $\mu$ g. The standard errors seen in the averages of 5 to 8 runs in which androsterone and etiocholanolone are separated from each other range from 2.5 to 6% of the amount of material recovered.

## 2. Preparation and Chromatography of Urine Extracts

The urines are hydrolyzed by boiling for 8 minutes with 15% volumes of hydrochloric acid and extracted with ether (Rubin *et al.*, 1954). After separation with the Girard's reagent (see page 85), the digitonin-nonprecipitable (or  $\alpha$ ) fraction of the ketonic extracts is prepared (see page 86). After determination of the 17-ketosteroid content by the Zimmermann reaction in terms of dehydroepiandrosterone, the remainder of the extract is transferred with benzene to a 3-ml. screw-capped vial and dried by heating in a water bath at  $90^{\circ}$ – $95^{\circ}\text{C}$ . under a stream of nitrogen.

Just before the urinary extract is placed on paper, it is dissolved in a volume of benzene such that 1.0 mg. or less of 17-ketosteroid (by Zimmermann reaction) is contained in 100  $\mu$ l. of solution. If the urinary extract contains 4–6.5 mg. of 17-ketosteroid, it is dissolved in 0.65 ml. of benzene. If fewer than 4 mg. are available for analysis, the extract is dissolved in 0.35 ml. of benzene, and the necessary changes in the routine technique are indicated in the proper places. Two 10- $\mu$ l. aliquots are taken from each urine extract for the quantitative Zimmermann determination. It must be known exactly how much 17-ketosteroid was put on each chromatogram in order to calculate back to the daily output of the various components.

The aliquots taken for analysis of the components are based on information as to the expected order of magnitude of the individual components. Four hundred microliters of solution are applied to each 6-cm. strip, and the chromatograms are allowed to develop for 24 hours in the heptane-propylene glycol system. All effluent is caught in a small beaker. Two hundred microliters are applied to a 2-cm. strip, and the chromatograms are developed for 72 hours in heptane-propylene glycol. (When the total volume of solution is only 0.35 ml., 300  $\mu$ l. are applied to the 6-cm. strip, and no 2-cm. chromatogram is prepared.) After the Zimmermann-positive zones are determined, the fractions are worked up as follows:

### *3. From the 24-Hour Chromatogram*

All material running faster than androsterone is eluted quantitatively from the paper with 95% ethanol and combined with the effluent. After evaporation of the ethanol, the material is transferred with benzene to a 15-ml. centrifuge tube and dried in the water bath under nitrogen. This material is then applied quantitatively to a 2-cm. strip impregnated with phenyl Cellosolve methanol, and allowed to run for 18 hours in the heptane-phenyl Cellosolve system. The effluent is again collected, and the Zimmermann values for the effluent are added to the figure determined from the zone ascribed to androst-2(or 3)-en-17-one, which is usually at the end of the strip. The other compounds determined in this chromatogram are 3 $\beta$ -chloroandrost-5-en-17-one, the component termed "II," a mixture of the etiocholanolone acetate and 3 $\alpha$ -acetoxy-5 $\beta$ -androst-9(11)-en-17-one, and some small amount of androsterone, remaining at the starting line, whose value can be added to that of the androsterone zone. Instead of being eluted quantitatively, the cutout zones are placed in 10 ml. of ethanol for 16 or more hours and aliquots taken for quantitative determination.

The androsterone zone is eluted quantitatively with ethanol. The ethanol is evaporated, and the residue is dissolved in 10 ml. of benzene. Aliquots of 0.25 and 0.5 ml. are taken for quantitative Zimmermann determinations, and 8.5 ml. are dried down in a 15-ml. centrifuge tube, under nitrogen. After the Zimmermann content of the material in the centrifuge tube has been determined, sufficient perbenzoic acid, dissolved in benzene, is added to the dried residue to oxidize the  $3\alpha$ -hydroxy- $5\alpha$ -androst-9(11)-en-17-one to the 9,11-epoxide. The solution of perbenzoic acid contains approximately 23 mg. per milliliter, and 0.2 ml. of perbenzoic acid solution is added for each 500  $\mu$ g. of combined androsterone and  $3\alpha$ -hydroxy- $5\alpha$ -androst-9(11)-en-17-one. The reaction is permitted to proceed for 5 hours at  $5^{\circ}$ – $7^{\circ}$ C. The material is then washed into a 125-ml. separatory funnel, with about 30 ml. of ethyl acetate. The ethyl acetate is washed with 5% sodium bisulfite, 5% sodium bicarbonate, and then with water until neutral to litmus. The ethyl acetate is removed *in vacuo*, the residue is transferred quantitatively with benzene to a 15-ml. centrifuge tube, and the benzene is removed. The residue is applied, in benzene solution, to a 2-cm. strip, and chromatographed for 24 hours in the heptane-propylene glycol system. Crystalline androsterone is used for a reference standard. The major zone, moving slower than androsterone which appears in these chromatograms, has been shown by infrared analysis to be the 9,11-epoxide of androsterone. Repeated studies of the perbenzoic acid oxidation have demonstrated that the amount of epoxide determined by the foregoing procedure represents  $81 \pm 2.5\%$  of the amount of  $3\alpha$ -hydroxy- $5\alpha$ -androst-9(11)-en-17-one present before oxidation. From this it is possible to calculate the amount of the compound excreted per day. The daily androsterone excretion, then, is the value calculated from the androsterone zone, minus the value of the  $\Delta^9(11)$ -unsaturated compound, and plus the daily value of the  $5\alpha$ -androst-2(or 3)-en-17-one.

The etiocholanolone zone is treated similarly to the androsterone zone. A 48-hour oxidation time is necessary for optimum oxidation of  $3\alpha$ -hydroxy- $5\beta$ -androst-9(11)-en-17-one. The chromatogram of the oxidized material is developed for 48 hours. The major zone appearing on oxidation of  $3\alpha$ -hydroxy- $5\beta$ -androst-9(11)-en-17-one is the 9(11)-epoxide. The oxidation product accounts for  $68 \pm 1.3\%$  of the starting material, under the conditions defined.

When the total amount of 17-ketosteroid is less than 4 mg. and the volume of benzene solution only 0.35 ml., the zone from the 24-hour chromatogram between the etiocholanolone zone and the starting line is eluted quantitatively. The eluted material is then chromatographed on a 2-cm. strip for 72 hours in heptane-propylene glycol.

#### 4. From the 72-Hour Chromatogram

The following components are obtained from analysis of the 72-hour chromatogram: 11-ketoandrosterone, 11-ketoetiocholanolone, and an unresolved residue which gives an atypical, brownish Zimmermann color. When any significant concentration of 11-hydroxylated 17-ketosteroids is present, they can also be seen and determined in this unresolved residual zone.

#### B. GRADIENT ELUTION METHOD OF LAKSHMANAN AND LIEBERMAN (1954)

For this procedure, Harshaw chromatographic alumina [A1-0109-P], which passes a No. 200 mesh sieve, is equilibrated in a desiccator with  $\text{NaBr} \cdot 2\text{H}_2\text{O}$  for a 5-day period so that the moisture content is between 7 and 8%. The column is prepared by gradually pouring 15 gm. of the prepared alumina into a benzene-filled tube containing a plug of absorbent cotton which had been tamped with a glass rod. Sufficient air pressure is applied so that an outflow rate of 2.5 ml. per minute is maintained. After the excess benzene has run out, the urinary extract, containing between 5 and 10 mg. of total 17-ketosteroids and dissolved in 10 ml. of benzene, is carefully poured onto the column. The total neutral fraction is usually suitable for the fractionation by this method, which means that neither Girard nor digitonin separation need be done. When this solution has percolated through the column a second 10-ml. portion of benzene is added. When the level of benzene is about 1–2 cm. above the top of the alumina, the gradient elution procedure is started. An apparatus for this purpose is described by Lakshmanan and Lieberman (1954). The initial volume of benzene in the mixing chamber ( $V_0$ ) is 700 ml., while the dropping funnel contains an ethanol-benzene mixture ( $C_0$ ) equal to 4% ethanol (v/v). The inflow rate into the mixing chamber ( $R_1$ ) should be 0.33 ml. per minute and the outflow ( $R_2$ ) 2.5 ml. per minute.

Twenty fractions, each 10 ml. in volume, followed successively by 40- to 5-ml. fractions and 35- to 10-ml. fractions are collected. The column is now washed twice with 10 ml. each of 2% ethanol-benzene, twice with 10 ml. of 4% ethanol-benzene, and finally with 10 ml. of 5% ethanol-benzene.

Each fraction is assayed for 17-ketosteroids using the Zimmermann reaction (see page 69) and the values obtained are plotted against the tube numbers. The identity of the individual fractions may be verified by infrared analysis.

## C. GRADIENT ELUTION METHOD OF KELLIE AND WADE (1957)

This method involves the hydrolysis of the 17-ketosteroid glucuronides with  $\beta$ -glucuronidase (prepared from *Patella vulgata*), followed by continuous extraction of the free 17-ketosteroids from their sulfate esters by continuous extraction of an acidified aqueous solution with ether. By this procedure the separated individual 17-ketosteroids may be quantitated as well as categorized as to sulfate and glucuronide conjugation.

The fresh urine after the addition of solid  $(\text{NH}_4)_2\text{SO}_4$  (50 gm./100 ml. of urine) is shaken in a separatory funnel until all the salt is in solution. The solution is extracted 3 times with  $\frac{1}{2}$  volume of 3:1 ether-ethanol mixture. The combined ether-ethanol layers are filtered and evaporated to dryness under reduced pressure. To remove the last traces of  $(\text{NH}_4)_2\text{SO}_4$  the dry gum is dissolved in absolute ethanol (about 1/10 of the original urine volume) and filtered into the vessel which will be used for the enzyme hydrolysis. The ethanol is evaporated to dryness by heating below  $40^\circ\text{C}$ . or in a stream of nitrogen.

The dried residue (from  $\frac{1}{2}$  of a 24-hour urine sample) containing the conjugates is dissolved in 0.5 *M* sodium acetate buffer and 200 mg. of  $\text{KH}_2\text{PO}_4$  added. Then 100 mg. of a powder derived from *Patella vulgata* according to the method of Dodgson and Spencer (1953) and containing 100,000 units is homogenized in 20 ml. of sodium acetate buffer and added to the solution containing the conjugates. An additional 5 ml. of buffer is used to wash the flask containing the enzyme and added to the mixture. Penicillin G (80,000 units) is added, hydrolysis carried out at  $40^\circ\text{C}$ . for 16 hours, the free steroids removed with benzene ( $2 \times 20$  ml. followed by  $1 \times 10$  ml.), centrifuged at 2000 r.p.m. for 2 minutes to break the emulsion, and the benzene layer removed with a pipet. The combined benzene extracts are washed with 1 *N* NaOH ( $3 \times 5$  ml.), then with water until the wash waters are neutral, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and the benzene removed. This fraction contains those 17-ketosteroids which were conjugated as glucuronides.

The aqueous layer, after benzene extraction, contains the sulfate esters of the 17-ketosteroids, is diluted to 100 ml., the pH adjusted to 1.0 with HCl, and the solution extracted continuously with ether in a liquid-liquid extractor for 72 hours. The ether layer is washed 3 times with 10-ml. volumes of 1 *N* NaOH, and finally with water to remove the alkali. The washed ether layer is dried over anhydrous sodium sulfate and the ether evaporated to dryness. This residue now constitutes the 17-ketosteroid fraction derivable from the sulfates.

The extracts are subjected to gradient elution using an alumina-adsorption column and a gradient of ethanol in benzene. The method is similar to that of Lakshmanan and Lieberman (1954). The excellency of the separation is illustrated by the fact that androsterone, etiocholanolone, 11-ketotiocholanolone, 11-ketoandrosterone, 11 $\beta$ -hydroxyetiocholanolone, and 11 $\beta$ -hydroxyandrosterone could be efficiently and sharply separated.

## VII. 17-Ketosteroids in Plasma and Serum

### A. METHOD OF MIGEON AND PLAGER (1955)

This method requires 25 ml. of plasma and consists of an ethanol extraction, evaporation of ethanol and solution of the residue in water, continuous extraction with ether at pH 0.8 for 48 hours, washing of the ether extract with sodium bicarbonate and water, chromatography on a Florisil column, removal of phenols with 1 *N* NaOH, paper chromatography, and quantitation of the dehydroepiandrosterone and androsterone by a micro Zimmermann method.

Recoveries of dehydroepiandrosterone and androsterone were determined to be 72 to 86% and a 20% variation could be expected in duplicate determinations. The individual steps in the procedure as suggested by the authors are recorded in Table V.

The significance of this method is not clear since no difference could be demonstrated between the values for plasma in men and women. For 15 normal adult males (age not specified) the dehydroepiandrosterone was reported to be 40.5(29-69)  $\mu$ g./100 ml. of plasma and that of androsterone 18(9-39)  $\mu$ g./100 ml. The levels were reported to vary somewhat with the menstrual cycle but were not significantly different from those of men (Migeon, 1954).

### B. METHOD OF SAIER *et al.* (1959)

Saier *et al.* (1959) have described a combined method for the determination of corticoids and 17-ketosteroids in serum based in part on the Nelson-Samuels (1952) and Eik-Nes *et al.* (1953) procedures for corticoids and the Burstein-Lieberman (1958) solvolysis method for the liberation of free dehydroepiandrosterone from its sulfate ester. Eight normal men (ages not specified) had a mean value of total 17-ketosteroids of 79  $\mu$ g. per milliliter of serum with a range of 19.9 to 122, while the

TABLE V

METHOD FOR MEASUREMENT OF DHA AND ANDROSTERONE  
IN PERIPHERAL VEIN PLASMA OF MAN<sup>a</sup>

- 
1. *Ethanol extraction*  
20 ml. plasma + 100 ml. EtOH: stir, centrifuge, remove EtOH extract.  
Wash protein residue with 50 ml. EtOH, centrifuge, add EtOH to first extract.  
Evaporate EtOH; add 25 ml. water.
  2. *Continuous extraction*  
Adjust pH to 0.8–1.0 with  $\text{H}_2\text{SO}_4$ .  
Extract for 24 to 48 hours with ether.
  3. *Washing of the extract*  
Reduce ether volume to approximately 150 ml.  
Wash with 10%  $\text{NaHCO}_3$  (10 ml.  $\times$  2) and water (20 ml.  $\times$  2).  
Distribute ether equally in two flasks and evaporate to dryness.
  4. *Column chromatography*  
Prepare two Florisil columns.  
Dissolve each half-extract in exactly 20 ml. of  $\text{CHCl}_3$  and pour over each column.  
Elute with 35 ml. of 2% MeOH in  $\text{CHCl}_3$ .
  5. *Removal of phenols*  
Continue the two eluates and wash with 1 *N* NaOH (10 ml.  $\times$  2) and water (20 ml.  $\times$  2).
  6. *Paper chromatography*  
Evaporate to dryness.  
Transfer extract onto paper, with DHA and androsterone alongside.  
Chromatograph in Bush light petroleum-methanol:water (85:15) system.  
Elute area of chromatogram corresponding to DHA and androsterone.
  7. *Micro Zimmermann reaction.*
- 

<sup>a</sup> From Migeon and Plager (1955).

dehydroepiandrosterone value was 38.2  $\mu\text{g}$ . per milliliter (range 15–101.0). Values for normal women were also reported but no data as to age or phase of menstrual cycle were included in the report. The fifteen normal women had a total 17-ketosteroid value of 94.5  $\mu\text{g}$ ./100 ml. of serum (range 22.6–162), while the mean value for dehydroepiandrosterone was 50.5 (range 3.0–116).

Serum is prepared from 25–30 ml. of whole blood and poured into a 50-ml. Erlenmeyer flask. It is recommended that serum be frozen within an hour of the blood collection and that the serum be analyzed within 1 week of freezing. The serum (10 ml.) is extracted 3 times with 15 ml. of redistilled chloroform and the aqueous portion used for the 17-ketosteroid determination.

*Extraction, Chromatography, and Solvent Partition of Serum  
17-Ketosteroids*

*1. Ethyl-acetate extraction*

- (a) Drain the aqueous portion obtained in Section VII, from the separatory funnel into a graduate.
- (b) Rinse the funnel with water and add the rinsings to the graduate to make the volume up to 16 ml.
- (c) Transfer to a 125-ml. Erlenmeyer flask and add an additional 16 ml. of water. Use this water to rinse the graduate. The total volume is now approximately 32 ml.
- (d) Adjust pH to 1.0 with a few drops of 18 *N* H<sub>2</sub>SO<sub>4</sub>.
- (e) Add 3.6 ml. of 18 *N* H<sub>2</sub>SO<sub>4</sub> to make a 2 *N* solution.
- (f) Add 40 ml. of ethyl acetate to the acidified emulsion, swirl, and mix well.
- (g) Transfer to 50-ml. round-bottomed centrifuge tubes, cap with foil, and centrifuge 10 minutes.
- (h) Decant the clear ethyl-acetate extract into a clean 125-ml. Erlenmeyer flask.
- (i) Wash the precipitated material 4 times with 10-ml. portions of ethyl acetate.
- (j) Centrifuge for 5 to 10 minutes after each wash and combine the ethyl-acetate extracts.

*2. Hydrolysis (solvolysis in ethyl acetate)*

Incubate the combined ethyl-acetate extracts from Part 1 above at 37°C. for 24 hours.

*3. Removal of phenolic, acidic, and pigmented material*

- (a) Wash the ethyl-acetate extract from Part 2 above once with an equal volume of 1% NaHCO<sub>3</sub>.
- (b) Filter into an Erlenmeyer flask and evaporate to dryness with an impinging air stream.
- (c) Add 5 ml. of repurified redistilled ethylene chloride to the dry residue. Rinse flask twice with 2.5 ml. of ethylene chloride to make a total volume of 10 ml. of ethylene chloride.
- (d) Transfer to a 50-ml. stoppered centrifuge tube.
- (e) Add approximately 20 pellets of NaOH, stopper, and shake 15 minutes in a shaker (Drekter *et al.*, 1952).

*4. Column chromatography*

- (a) Prepare a column (10 mm. in diameter) with 3 gm. of Florisil. Tap Florisil into column.
- (b) Wash column with 10 ml. of ethylene chloride and discard.



- (c) Filter the ethylene-chloride extract from Part 3 through a No. 1 Whatman filter paper directly onto the column. Rinse the centrifuge tube with 2.5 ml. of ethylene chloride and add to the column.
- (d) Elute with 35 ml. of 2% methanol in redistilled  $\text{CHCl}_3$  and collect the entire 47 ml. into an Evelyn colorimeter tube or flask.
- (e) Evaporate to dryness with an impinging air stream.

5. *Solvent partition*

- (a) Take up residue from Part 4 above in 3 ml. of 70% ethanol.
- (b) Add 3 ml. of hexane and shake 15 seconds.
- (c) Centrifuge and remove hexane with suction.
- (d) Repeat (b) and (c) twice.
- (e) Add 3 ml. of water to the ethanol extract and 6 ml. of repurified ethylene chloride.
- (f) Shake well and transfer to a calibrated 15-ml. centrifuge tube.
- (g) Rinse the Evelyn colorimeter tube with an additional 1 ml. of ethylene chloride and add to the calibrated tube.
- (h) Stir and centrifuge 10 minutes.
- (i) Remove aqueous ethanol layer with suction (total volume remaining, 7.5 ml.).
- (j) Pipet 2.5 ml. of the remaining 7.5 ml. of ethanol-chloride extract into a standard test tube and evaporate to dryness with air. This portion is used for determination of dehydroepiandrosterone by a micromodification of the Allen *et al.* (1950) method.
- (k) The remaining 5-ml. portion of ethylene chloride is transferred to an Evelyn colorimeter tube, the centrifuge tube being rinsed with an additional 0.5 ml. of ethylene chloride. The contents of the Evelyn tube are evaporated to dryness with an air stream. This portion is used for determination of total 17-ketosteroids by the micro Zimmermann method.

C. METHOD OF GARDNER (1953)

This method for total 17-ketosteroids in plasma involves treatment with acid to liberate the free steroids, column chromatography using Florisil, and a Zimmermann method for the quantitation. Normal adult (age not specified) men's plasma had values varying from 40 to 130  $\mu\text{g.}/100$  ml. of plasma and normal women had plasma values between 25 to 100  $\mu\text{g.}/100$  ml. Patients with untreated congenital adrenal hyper-

plasia had elevated values varying from 80 to 360  $\mu$ g. An adrenalectomized-oöphorectomized woman had no detectable amount of 17-ketosteroids.

Plasma (6–10 ml.) is boiled with 25 ml. of 10% HCl on a hot plate for 12 minutes. The flask is cooled and the contents transferred to a 250-ml. centrifuge tube. The contents are stirred with 25 ml. of ether and centrifuged to break the emulsions. The ether is removed and the process repeated 3 more times. The combined ether layers are washed with three 25-ml. portions of 10% NaOH and finally with water to remove all the alkali. The ether is removed by evaporation, the residue dissolved in 20 ml. of 70% ethanol, and the aqueous-ethanol solution washed 3 times with 15 ml. of hexane. The aqueous-ethanolic solution is diluted with 10 ml. of distilled water and extracted 3 times with 15-ml. portions of chloroform. The combined chloroform extracts were reduced to a volume of 12 ml. Three grams of Florisil were placed in a chromatographic column of 10 mm. inside diameter and the column washed with 30 ml. of chloroform followed by addition of the 12 ml. of the sample in chloroform. The column was developed with an additional 35 ml. of chloroform and approximately 47 ml. of eluate collected. The eluate is concentrated to dryness and used for analysis using a micro Zimmermann procedure.

### VIII. The Zimmermann Reaction

#### A. METHOD OF CALLOW *et al.* (1938)

This method is described in Section V, F.

#### B. METHOD OF HOLTORFF AND KOCH (1940)

The reagents consist of a 2% solution of *m*-dinitrobenzene in 95% redistilled ethanol and 5.00 *N* aqueous KOH (electrolytic grade). The latter solution should be protected with a paraffin seal to prevent any significant accumulation of carbonate.

#### *Procedure*

Into a photometer tube are accurately measured 0.2 ml. of solution or 0.2 ml. of redistilled 95% ethanol for blank, 0.2 ml. of *m*-dinitrobenzene solution, and 0.2 ml. of 5.0 *N* KOH solution. The tubes are then corked, gently shaken, and placed in a water bath for 90 minutes at  $25^{\circ} \pm 0.2^{\circ}\text{C}$ . At the end of 90 minutes the solution is diluted with 10 ml. of redistilled

95% ethanol. After the outside of the tube is dried and polished, it is placed in a rack for 3 minutes, and the optical density is measured, after the zero density point of the photometer is set with the blank. The 17-ketosteroid content of the test material is determined from the  $E_{5200}$  value by reference to a calibration curve constructed from measurements with androsterone or dehydroepiandrosterone. It has frequently been found advisable to run standards with each test run.

A number of 17-ketosteroid methods have employed a procedure involving the extraction of the pink chromogen with an organic solvent. Reference to the method of Peterson and Pierce (1960) has been made on page 65. Other methods using this principle include those of Cahen and Salter (1944), Zimmermann and Anton (1952), Rupert (1952), Masuda and Thuline (1953), and Werbin and Ong (1954).

## IX. Preparation of Neutral Ketonic Fractions

For certain procedures it is highly desirable to prepare the 17-ketosteroid fraction in as purified a state as possible. A case in point is the analysis of the individual 17-ketosteroids by paper chromatography (see page 72). Girard and Sandulesco (1936) suggested certain reagents which can be used for this purpose. One of these reagents, Girard reagent T (trimethylaminoacetohydrazide chloride), has been used extensively since it reacts with ketone groups leading to water-soluble products, while the nonketonic compounds remain lipid soluble and can be separated by a suitable solvent. The free 17-ketosteroids may be regenerated by hydrolysis with mineral acid followed by extraction with a lipid solvent. Two procedures which have been used successfully for urinary 17-ketosteroids are described in the following sections.

### A. METHOD OF TALBOT *et al.* (1940)

The dry residue of the total neutral fraction derived from a liter of urine is dissolved in 4 ml. of 95% ethanol, and, after addition of 0.5 ml. of glacial acetic acid and 0.5 gm. of Girard's reagent T, the solution is refluxed for 1 hour on a water bath. After the solution has been cooled and 40 gm. of ice added, 3 ml. of 2 *N* sodium hydroxide are added, and the mixture is extracted 4 times with 40-ml. portions of ethyl ether. The combined ethereal extracts, after being washed 3 times with 20-ml. portions of water, are evaporated to dryness. This residue constitutes the nonketonic neutral fraction. To the water washings of the above ether extract 1 ml. of concentrated sulfuric acid and 20 ml. of

ethyl ether are added. After the mixture has stood for at least 2 hours at room temperature, 1 ml. of concentrated sulfuric acid is added, to facilitate the extraction of the ketones, and the mixture is extracted 4 times with 40-ml. portions of ether. The combined ethereal extracts are washed with 20-ml. portions of water until the wash water is neutral in reaction. The ether layer is evaporated to dryness, and the residue constitutes the neutral ketonic fraction.

#### B. METHOD OF PINCUS AND PEARLMAN (1941)

The total neutral residue from 1 liter of urine is thoroughly dried in a Pyrex test tube over calcium chloride in a vacuum desiccator; 0.5 ml. glacial acetic acid and approximately 100 mg. of Girard's reagent T are added. The tube is loosely stoppered with cork wrapped in aluminum foil and placed in an oil bath at 90° to 100°C. for 20 minutes. The tube is cooled, 15 ml. of ice water are added, and the reaction mixture is immediately transferred to a small separatory funnel. Sufficient 10% NaOH is added to neutralize 9/10 of the acetic acid. The mixture is extracted with three 20-ml. portions of ether. An aqueous wash is combined with the main aqueous fraction. The layer contains the nonketonic compounds and may be discarded. The aqueous fraction which contains the ketonic compounds (17-ketosteroids included) is acidified with 3 ml. of concentrated hydrochloric acid, allowed to stand at room temperature for 2 hours, and then extracted 3 times with 20-ml. portions of ether. The ether is washed with 10 ml. of 2.5% sodium carbonate and 3 times with 10-ml. portions of water, or until the wash water is neutral in reaction. The ether is evaporated to dryness, and the residue constitutes the neutral ketonic fraction.

#### X. Preparation of the $\beta$ -17-Ketosteroid Fraction

The alcoholic group at position 3 in 17-ketosteroids may be either in the *cis* or *trans* position compared to the angular methyl group at carbon 10. Conventionally, the *cis* form has been designated as  $3\beta$  while the 17-ketosteroid having the 3-hydroxy *trans* configuration is designated as the  $3\alpha$ -hydroxy form. Digitonin, a steroid saponin, under certain controlled conditions usually precipitates those steroids having a  $3\beta$ -hydroxy configuration and in the 17-ketosteroid series may be designated as  $\beta$ -17-ketosteroids. Dehydroepiandrosterone, present both in urine and plasma, is the most significant member of the group, and relatively specific,

simple, and efficient methods are described in Section II. A number of useful methods involving digitonin precipitation are described in this section and may be of value for special studies.

#### A. METHOD OF FRAME (1944)

The total neutral fraction or the neutral ketonic fraction from 2 liters of urine (containing about 15 mg. of 17-ketosteroids) is dissolved in absolute ethanol and transferred quantitatively to a calibrated 15-ml. centrifuge tube and warmed. To the ethanol solution is added a warm solution of digitonin in such proportions of absolute alcohol and water that the final concentration of digitonin is 1% in 90% ethanol. At least 14 mg. of digitonin should be allowed for each milligram of 17-ketosteroid expected in the test solution. The tube is stoppered, centrifuged for 10 minutes at about 200 r.p.m., and the supernatant poured off into a 250-ml. separatory funnel. The precipitate, which contains the beta fraction, is washed 3 times with 10-ml. portions of ether, with stirring and centrifuging after each addition. The ether washings are added to the supernatant on the separatory funnel. The combined ether extracts are washed 3 times with 25-ml. portions of water, the ether solution is drawn into a flask, evaporated under reduced pressure, and dried in a desiccator over calcium chloride. This fraction contains the material which is not precipitable by digitonin and which is designated the alpha fraction.

The precipitate remaining in the centrifuge tube (beta fraction) is dissolved in 0.5 ml. of dry pyridine and heated for 3 minutes in a hot water bath. After the mixture is cooled, 10 ml. of anhydrous ether are added to precipitate the digitonin, and the tube is centrifuged for 10 minutes. The supernatant, containing the beta fraction, is poured into a 250-ml. separatory funnel, and the pyridine-ether treatment of the precipitate is repeated. The remaining precipitate is washed twice with 10 ml. of ether, with stirring and centrifuging each time. The combined ether-pyridine solution is washed 3 times with 10-ml. portions of 2 *N* sulfuric acid to remove the pyridine and 3 times with 25-ml. portions of water to remove the sulfuric acid. The ether solution is evaporated to dryness, the residue constitutes the  $\beta$ -ketonic neutral fraction.

When the starting volume is greater than 3 ml., that is, when more than 30 mg. of digitonin are required to precipitate the beta fraction, it is preferable to carry out the separations in a 50-ml. centrifuge tube. The procedure here is identical with that outlined above, except that 25-ml. portions of ether, 1.0- or 1.5-ml. portions of pyridine, and 20- to 25-ml. portions of acid are used.

### B. METHOD OF BUTT *et al.* (1948)

An amount of urine containing 1.0–1.5 mg. of 17-ketosteroids is transferred to a graduated centrifuge tube and evaporated to dryness under reduced pressure. Warm digitonin solution [0.75 ml. of 1% solution in 90% (v/v) ethanol] is added, and the mixture is quickly heated to boiling in order to dissolve as much of the material as possible. The tube is stoppered and left in a refrigerator overnight. A total of 10 ml. of peroxide-free ether is then added in small portions, with stirring after each addition, and the precipitated digitonin and digitonides are allowed to flocculate before the last 2–3 ml. are added. After centrifuging, the supernatant is decanted into a separatory funnel, and the precipitate is washed 3 times with 5-ml. portions of ether, with stirring and centrifuging each time. The combined supernatants are washed 3 times with 5-ml. portions of water, and are then evaporated to dryness under reduced pressure. The residue constitutes the  $\alpha$ -ketonic fraction. The precipitate, containing the  $\beta$ -ketonic substances, is dissolved in 0.25 ml. of dry pyridine, warmed to 60°–70°C. for 3 minutes, cooled, and 5 ml. of ether added in portions, stirred, and the precipitate is allowed to flocculate. The mixture is centrifuged, and the precipitate is again treated with pyridine and ether, and the final residue washed twice with 5-ml. portions of ether. The combined extracts are washed twice with 5-ml. portions of water, and then evaporated to dryness under reduced pressure. The residue is the  $\beta$ -ketonic fraction.

## XI. Miscellaneous Methods

Polarographic methods for the determination of 17-ketosteroids have been shown to be accurate and dependable but have not enjoyed wide popularity. Wolfe *et al.* (1940) and Hershberg *et al.* (1941) reacted 17-ketosteroids with the Girard reagent T and the specific half-wave potential of hydrazones so formed used for the determination. This method was further developed by Barnett *et al.* (1946a, b), Butt *et al.* (1948), and by Butt (1950). Good agreement was found between values obtained by the Zimmermann methods and those obtained by the polarographic technique as evidenced by a correlation coefficient of 0.87 for the values obtained on the same urines by the two methods.

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## Chapter 3

# Progesterone

JOSEF ZANDER

I. Historical Survey .....	91
II. Extraction and Preliminary Purification .....	96
A. Extraction with Organic Solvents, Removal of Proteins and of Lipids .....	96
B. Extraction with Ether after Pretreatment with Alkali .....	98
C. Separation of Ketones with Girard's Reagent T .....	99
III. Isolation of Progesterone from Purified Extracts .....	100
A. Countercurrent Distribution .....	100
B. Column Chromatography .....	101
C. Paper Chromatography .....	103
IV. Quantitative Estimation of Isolated Progesterone .....	106
A. Spectrophotometric Estimation as $\Delta^4$ -3-Ketosteroid .....	106
B. Polarographic Estimation as Girard Complex .....	107
C. Spectrophotometric Estimation of Progesterone Derivatives .....	108
D. Sulfuric Acid-Ethanol Reaction .....	111
E. Fluorescence Reactions .....	111
F. Application of the Color Correction Equation of Allen .....	112
V. Additional Identification of Progesterone .....	115
A. Color Reactions .....	115
B. Sulfuric Acid Reaction .....	116
C. Formation of Derivatives .....	117
D. Infrared Absorption Spectrum .....	117
VI. Reliability of Different Methods .....	118
A. Specificity .....	118
B. Accuracy .....	119
C. Precision .....	122
D. Sensitivity .....	122
VII. Description of Recommended Methods .....	123
A. Method of Zander and Simmer (1954) .....	123
B. Method of Short (1958) .....	127
C. Method of Oertel, Weiss, and Eik-Nes (1959) .....	128
VIII. Application of Methods .....	130
A. Tissue .....	130
B. Plasma .....	132
IX. Suggestions for Further Development of Methods .....	133
References .....	134

## I. Historical Survey

The pioneering work of Prenant of Nancy, Fränkel, Ancel and Bouin, Loch, Hisaw, and also of Corner established the fact that the corpus

luteum of the ovary produces a hormone which, besides having other effects, is responsible for the secretory transformation of the uterine mucosa. On the basis of these investigations, Corner and Allen (1929) developed a relatively simple biological assay for the hormone. This method proved to be of fundamental importance for the isolation of the genuine hormonal substance: it provided a quantitative assay of material obtained by chemical extraction procedures.

Corner and Allen in 1929 obtained active extracts from corpora lutea of sows. Their method was based originally on a principle employed for the first time for luteal tissue by Herrmann (1915), consisting essentially in the extraction of the lipids by hot alcohol and the subsequent precipitation of the phospholipids from an ethereal solution by the addition of acetone. Within a short time after its publication the method was improved, and three groups, Fels and Slotta (1931) in Breslau, Fevold and Hisaw (1932; Fevold *et al.*, 1932) in Wisconsin, and Allen (1932) in Rochester, New York, were able to prepare highly active crystalline material from pig corpora lutea.

After further improvements in the purification techniques, Butenandt *et al.* (1934b; Butenandt and Westphal, 1934a) in Danzig and Berlin, Hartmann and Wettstein (1934a, b) in Basle, Slotta *et al.* (1934) in Breslau, and Wintersteiner and Allen (1934; Allen and Wintersteiner, 1934) in New York and Rochester, New York, independently succeeded in the isolation of the corpus luteum hormone, later named progesterone. In the same year the structure was elucidated and shown to be correct by several synthetic methods (Butenandt *et al.*, 1934a; Butenandt and Westphal, 1934b; Fernholz, 1934; Butenandt and Schmidt, 1934).

The methods developed by the groups just mentioned contained numerous valuable observations for future efforts toward devising chemical procedures for the determination of progesterone. The first attempt in this direction was made by Reynolds and Ginsburg (1942). Starting with the extraction and purification procedure of Allen (1932), they used, for the quantitative measurement, the maximal ultraviolet absorption at  $240\text{ m}\mu$  which progesterone and other  $\Delta^4$ -3-ketosteroids exhibit. Although it was possible to detect 0.1–5.0 mg. progesterone added to 10 ml. of serum the method suffered from a lack of specificity and sensitivity. It failed to live up to expectations because at that time satisfactory microanalytical procedures for the separation of progesterone from other  $\Delta^4$ -3-ketosteroids were not available.

At the same time, the determination of urinary pregnanediol received great attention (see Chapter 4). The routine estimation of this progesterone metabolite was first introduced by Venning (1937, 1938) and

her work provided, although in an indirect fashion, new knowledge about progesterone as a precursor of pregnanediol.

For a long time the quantitative determination of progesterone itself in blood or tissues remained in the domain of biological assays. It early became evident that the sensitivity of the Corner-Allen test or any of its modifications could not be considered adequate for general routine use. But the biological microassay presented by Hooker and Forbes (1947), characterized by far greater sensitivity for progesterone, stimulated many research projects in this area. The relatively high values for blood progesterone found by this bioassay method stimulated a number of investigators to develop chemical methods for the determination of progesterone.

Haskins (1950), returning to the earlier efforts of Reynolds and Ginsburg (1942), introduced a simplified ether extraction of the plasma without further purification. For routine determinations of progesterone in plasma this was not successful, but Haskins employing this method following intravenous injection of progesterone made some notable observations which remain valid up to the present time. In the same year he tried for the first time to isolate progesterone by paper chromatography from commercially prepared oily vehicles employing a "monophasic" solvent system of 80% methanol (Haskins *et al.*, 1950). With this system progesterone ran essentially with the solvent front, but could be separated from the oil satisfactorily.

At this stage it was obvious that the further development of micro-analytical chemical methods depended first of all upon the elaboration of reliable and more sensitive techniques for the separation of progesterone.

Progressing in this direction a new method to measure progesterone in blood was described by Butt *et al.* (1951) who presented definite improvements and simplifications of the extraction and preliminary fractionation procedure of Allen (1932). For the subsequent isolation of progesterone a partition chromatography on a Hyflo Supercel column was introduced, followed by a polarographic quantitative measurement of progesterone as the Girard complex. Values obtained for the progesterone in placental blood with this technique were confirmed later by other methods, but for the measurement of progesterone in peripheral blood the method still lacked sensitivity.

A year later column chromatography and countercurrent distribution methods were successfully applied by Salhanick *et al.* (1952), Pearlman and Cerceo (1952), as well as by Diczfalussy (1952) for the isolation of progesterone from the human placenta.

TABLE I  
SURVEY OF MICROANALYTICAL ROUTINE METHODS FOR THE DETERMINATION OF PROGESTERONE

Authors	Extraction	Preliminary purification	Isolation of progesterone	Quantitative estimation
Reynolds and Ginsburg (1942)	Ethanol	Partition in organic solvents. Removal of lipids in cold 70% methanol	—	UV absorption at 240 m $\mu$
Haskins (1950)	Ether	—	—	UV absorption at 240 m $\mu$
Butt <i>et al.</i> (1951)	Ethanol-ether (3:1 v, v)	Partition in organic solvents. Removal of lipids in cold 70% methanol	Partition chromatography on Hyflo Supercel	Polarographic as Girard complex
Edgar (1953a); Zander and Simmer (1954); Raeside and Turner (1955); Wiest (1959)	Ethanol-ether (3:1 v, v)	Partition in organic solvents. Removal of lipids in cold 70% methanol	Paper chromatography	UV absorption at 240 m $\mu$
Pearlman and Cerreo (1952, 1953)	Ether after pretreatment with alkali	Partition in organic solvents. Separation of ketones with Girard's reagent T	Countercurrent distribution	UV absorption of thiosemicarbazide derivative at 300 m $\mu$
Haskins (1954)	Ether after pretreatment with alkali	Partition in organic solvents. Separation of ketones with Girard's reagent T	Paper chromatography	UV absorption at 240 m $\mu$

Hinsberg <i>et al.</i> (1956)	Ether	Chromatography on $\text{Al}_2\text{O}_3$	Chromatography of 2,4-dinitrophenylhydrazine derivative on $\text{Al}_2\text{O}_3$	UV absorption of 2,4-dinitrophenylhydrazine derivative at 380 $\text{m}\mu$
Loy <i>et al.</i> (1957)	Ethanol	Chromatography on $\text{Al}_2\text{O}_3$	Countercurrent distribution	UV absorption at 240 $\text{m}\mu$
Short (1958)	Ether after pretreatment with alkali	Partition in organic solvents	Paper chromatography	UV absorption at 240 $\text{m}\mu$
Sommerville and Deshpande (1958)	Ether-methylene chloride (4:1 v/v) after pretreatment with alkali	Chromatography on $\text{Al}_2\text{O}_3$	—	UV absorption of isonicotinic acid hydrazine derivative at 380 $\text{m}\mu$
Gorski <i>et al.</i> (1958)	Ethyl acetate; benzene-ethyl acetate	Partition in organic solvents. Removal of lipids in cold 70% methanol	Paper chromatography	UV absorption at 240 $\text{m}\mu$
Oertel <i>et al.</i> (1959)	Ethanol	Partition in organic solvents. Removal of lipids in cold 70% methanol	Paper chromatography	UV absorption of sulfuric acid chromogens at 290 $\text{m}\mu$
Simmer and Simmer (1959)	Ethanol	Partition in organic solvents. Removal of lipids in cold 70% methanol	Paper chromatography	UV absorption of thiosemicarbazide derivative at 300 $\text{m}\mu$

It was the work of Zaffaroni and his co-workers (1950) and of Bush (1952) on the paper chromatographic separation of steroids, which expedited further developments. Edgar (1953a), and independently Zander and Simmer (1954), starting with the somewhat modified extraction and purification procedure of Butt *et al.* (1951), isolated progesterone from the purified extracts using the paper chromatographic systems of Bush. For the quantitative measurement the absorbancy at  $240\text{ m}\mu$  was used as proposed by Reynolds and Ginsburg (1942). Subsequently these methods found a broader practical application for the determination of progesterone in blood and tissues under a variety of physiological conditions. Zander (1954) succeeded finally in the quantitative isolation of progesterone from human peripheral blood using infrared spectrophotometry for identification. It was found in these investigations that the progesterone concentrations in the plasma of women during the second half of pregnancy varied between  $3.9$  and  $26.8\text{ }\mu\text{g.}$  in  $100\text{ ml.}$  of plasma with an average of  $14.2\text{ }\mu\text{g.}$  These results indicated the range of sensitivity required for methods of progesterone determination. These studies also settled the old controversy about the relatively high blood values that had been found with the Hooker-Forbes test.

Recent progress in the development of physicochemical methods for the routine determination of progesterone in blood and tissues has been rapid and extensive. Many new facts have been reported so that a first review of the field and the available methods seems justified.

Table I summarizes the techniques that have been employed for extraction, preliminary purification, isolation, and quantitative estimation of progesterone. The various methods will be discussed in detail in the subsequent sections.

## II. Extraction and Preliminary Purification

### A. EXTRACTION WITH ORGANIC SOLVENTS, REMOVAL OF PROTEINS AND OF LIPIDS

In these studies, leading to the isolation and identification of progesterone, as they have been surveyed in Section I, the lipids from the corpus luteum tissue were usually extracted by hot ethanol. The precipitated proteins were discarded and the alcoholic extracts, after evaporation, were taken up in ether. This alcohol extraction is still successfully applied in routine methods for the estimation of progesterone in plasma and tissues (Loy *et al.*, 1957; Oertel *et al.*, 1959; Simmer and Simmer, 1959). A mixture of ethanol and ether (3:1 v/v), well known for a long time in analytical chemistry of lipids, also proved to be ex-

tremely successful for the extraction of progesterone-containing material (Butt *et al.*, 1951; Edgar, 1953; Zander and Simmer, 1954; Raeside and Turner, 1955). After removal of the precipitated proteins the extracts are evaporated *in vacuo* to about 2–3 ml. and are diluted with water and then extracted with ethyl acetate or benzene (Zander and Simmer, 1954).

For the determination of progesterone in plasma and tissue, other solvents such as ether (Haskins, 1950; Hinsberg *et al.*, 1956), ether-methylene chloride (4:1 v/v) (Sommerville and Deshpande, 1958), acetone (Pearlman and Thomas, 1953), ethyl acetate and a mixture of this solvent with benzene (Gorski *et al.*, 1958) have been proposed.

After the extraction with organic solvents practically all the lipids are in the solvent phase and thus it becomes necessary to separate the relatively fat-soluble progesterone from the lipid fraction. Subsequently it will be possible to isolate progesterone by chromatography in a state of purity suitable for microdetermination. Experience has shown that the immediate column or paper chromatography of inadequately prepurified extracts leads to insufficiently pure progesterone fractions which cannot be properly estimated quantitatively. The chromatographic procedures yield at best only some preliminary purification and need to be supplemented by other methods of separation (see Section III, B).

In the course of his very early investigations, aimed at the preparation of purified progestins, Allen (1932) developed useful methods for the removal of contaminating lipids from the extracts. He found that a simple distribution between 70% methanol and petroleum ether yields a remarkable separation, which leaves the major portion of progesterone in the alcoholic phase. Far more effective however was the removal of lipids by freezing a 70% methanol solution of the crude extract.

This procedure proved to be valuable also for routine determinations of progesterone in biological material (Butt *et al.*, 1951; Edgar, 1953b; Zander and Simmer, 1954; Raeside and Turner, 1955; Gorski *et al.*, 1958; Oertel *et al.*, 1959). For this method the extracts have to be relatively protein free, because, otherwise, the freeze-precipitation of the neutral fats might be disturbed.

The evaporated lipid extracts are dissolved in 70% methanol at about 35°C. This procedure, because of the slow solubility of fat in 70% methanol, takes several minutes. (It is not advisable to dissolve the fatty extracts in absolute methanol and to dilute it with water.) In the methanolic solution the neutral lipids are precipitable at  $-10^{\circ}$  to  $-15^{\circ}$ C. and can be separated by centrifugation in a refrigerated centrifuge at about  $-15^{\circ}$ C. followed by decantation. Best results are obtained with a high-speed centrifuge, but 2000 to 3000 r.p.m. will be found reasonably satisfactory. Progesterone is then extracted with light petroleum ether after

dilution of the methanolic solution with water. [A low boiling petroleum ether fraction (35°–45°C.) is preferable, because the small amounts of fat which may have remained dissolve more easily in the higher boiling fractions.]

From our experience in the laboratory even in extracts of high fat content one can recover 96% of added radioactive progesterone with a single freeze-precipitation. The petroleum ether extracts are suitable for further separation procedures including the sensitive paper chromatographic systems of Bush (1952). As Kaufmann and Zander (1956) demonstrated, the method can be applied even to fat tissues.

#### B. EXTRACTION WITH ETHER AFTER PRETREATMENT WITH ALKALI

For the extraction of progesterone from the corpus luteum of the whale, Prelog and Meister (1949) developed a method which Ogata and Hirano (1933) originally used to obtain androgenically active testis extracts. To minced tissue is added an equal volume of 5% NaOH, so as to secure a final concentration of 2.5% NaOH in the mixture, which is subsequently covered with peroxide-free ether. The same principle has been used successfully for the isolation of progesterone from human placental tissue (Salhanick *et al.*, 1952; Pearlman and Cerceo, 1952; Diczfalusy, 1952; Noall *et al.*, 1953).

When ether was added immediately following the addition of NaOH to the tissue mash, the ether extracts were later found to contain considerable amounts of fatty material; Noall *et al.* (1953) observed that far purer extracts may be obtained if the NaOH treatment proceeds without ether overlay, and when the ether extraction is started 2 days after NaOH addition. With this modification Haskins (1954) developed a routine determination procedure for progesterone determination in human placenta. But, nevertheless, this much improved procedure still requires further thorough purification steps before the quantitative estimation of progesterone can be done.

One essential point was learned from the alkali treatment studies, namely the recognition that progesterone is not nearly as sensitive to alkali as formerly assumed. Comparing the quantitative results of experiments in which placental tissue was extracted with ether after alkali treatment (Salhanick *et al.*, 1952; Pearlman and Cerceo, 1952; Diczfalusy, 1952; Noall *et al.*, 1953; Haskins, 1954), with the results of those studies in which the alcohol-ether extraction was done without preliminary alkali treatment (Zander and von Münstermann, 1956) one sees that the differences are negligible.

Sommerville and Deshpande (1958) and also Short (1958) adopted



the ether extraction technique after alkali treatment in modified form for the extraction of progesterone from plasma. The first group of workers added to plasma an equal volume of 0.33 *N* NaOH and extracted after 5 minutes of mechanical stirring with an ether-methylene chloride mixture (4:1 v/v). The latter author added NaOH pellets or a 5% NaOH solution to give a final concentration of 0.5% NaOH in the plasma. After thorough stirring the plasma is extracted with ether. After evaporation of the ether one obtains a fairly well-purified fraction by a simple distribution procedure between light petroleum and 70% methanol, which is suitable for further manipulation with sensitive chromatographic separation techniques.

We conducted a comparative study (Zander, 1959, unpublished results) with 19 samples of pooled human cord plasma, 10 of which were extracted according to Short after alkali treatment, while 9 samples were extracted without pretreatment with alkali according to the method of Zander and Simmer with ethanol-ether (3:1 v/v). The results were as follows. Both methods yielded the same average progesterone concentration (32.2  $\mu$ g./100 ml. plasma). The precision (see Section VI, C) was better with alkali treatment (S.D.  $\pm$  2.1) than without alkali treatment (S.D.  $\pm$  6.4). [We have recently improved the precision of the latter method (see Table VI).]

It can be stated without hesitation that the ether extraction after alkali pretreatment is of great value for progesterone determination, especially in plasma. The method is simple and practicable and gives results equivalent to those obtained with the methods described in Section II, A. The distinct disadvantage is that the alkali technique does not permit simultaneous assays for steroids which are more sensitive to alkali than is progesterone.

### C. SEPARATION OF KETONES WITH GIRARD'S REAGENT T

The condensation of ketones with reagent T of Girard (Girard and Sandulesco, 1936), often used in working with steroids, was useful for the separation of the ketonic fraction before isolating progesterone. Prelog and Meister (1949), by use of this technique, were able to obtain a remarkable degree of purity in working with the concentrated ether extracts of whale corpora lutea treated with alkali. Repeated reactions with Girard's reagent yielded 8.53 gm. of a ketonic fraction from 119 gm. of extract. The use of this technique permitted the isolation of progesterone from large quantities of human placentas (Pearlman and Cerceo, 1952; Diezfulusy, 1952; Noall *et al.*, 1953; Haskins, 1954). Pearlman and Thomas (1953) made use of this separation of the ketonic fraction for

the isolation of progesterone from pooled human cord blood. The method, however, has yet to find a place in the procedure for the routine micro-analytical determination of progesterone.

### III. Isolation of Progesterone from Purified Extracts

#### A. COUNTERCURRENT DISTRIBUTION

Countercurrent distribution as introduced by Craig *et al.* (1945) has been applied to the isolation of progesterone from human placenta by Pearlman and Cerceo (1952) and Diczfalusy (1952), from umbilical venous blood by Pearlman and Thomas (1953), and, furthermore, from luteal tissue by Loy *et al.* (1957).

Pearlman and Cerceo (1952) treated the extracted material after preliminary purification with reagent T of Girard and Sandulesco (1936). The ketonic material thus obtained was subjected to an 8-transfer distribution. The solvent system employed was petroleum ether : 70% methanol (3:1 v/v). The partition coefficient (*K*) of progesterone in this system was found to be 1.0. The same system was chosen by Loy *et al.*

TABLE II  
PARTITION COEFFICIENT (*K*) OF PROGESTERONE  
AND OTHER  $\Delta^4$ -3-KETOSTEROIDS

Compound	Solvent system			
	Petroleum ether: 70% methanol (equal volumes) <sup>a</sup>	Petroleum ether: 34.5% ethanol (equal volumes) <sup>b</sup>	<i>n</i> -Hexane: ethanol (equal volumes) <sup>b</sup>	<i>n</i> -Hexane: ethanol (equal volumes) <sup>c</sup>
Progesterone	0.33	3.55	1.03	3.35
Pregna-4,11-diene-3,20-dione	—	5.15	1.13	—
20 $\alpha$ -Hydroxypregn-4-en-3-one	—	—	—	0.64
20 $\beta$ -Hydroxypregn-4-en-3-one	—	—	—	1.44
Androst-4-ene-3,17-dione	0.07	—	—	0.56
Testosterone	0.05	0.28	—	0.16
Deoxycorticosterone	0.03	0.21	—	—
17 $\alpha$ -Hydroxy progesterone	—	<0.02	—	0.14

<sup>a</sup> Data in this column reported by Pearlman (1954).

<sup>b</sup> Data in this column reported by Diczfalusy (1952).

<sup>c</sup> Data in this column obtained in the author's laboratory (Zander, 1959, unpublished results).

(1957). However, for a complete isolation of progesterone, this system proved to be inadequate. Further purification of the contents of the central separatory funnels, by chromatography or other methods, was found to be necessary. A better separation was obtained with a 24-transfer distribution between equal volumes of petroleum ether and 34.5% ethanol, as Diczfalusy (1952) demonstrated with placental extracts which had been purified according to the methods of Allen (1932) or of Prelog and Meister (1949). The partition coefficient ( $K$ ) of authentic progesterone in this system is 3.55. Diczfalusy (1952) observed also that the system  $n$ -hexane : 48.4% ethanol (1:1 v/v) [partition coefficient ( $K$ ) of progesterone 1.03] is suitable.

Except for Loy *et al.* (1957) the countercurrent distribution has not yet been applied for routine determinations. The chromatographic techniques are more practical and less time consuming. However, for supplementary characterization and additional purification, countercurrent distribution may be of great help, particularly when working with pooled extracts.

Partition coefficients of some less polar  $\Delta^4$ -3-ketosteroids in comparison to progesterone are listed in Table II.

### B. COLUMN CHROMATOGRAPHY

Several authors have taken advantage of the column chromatography as a supplementary method to isolate progesterone. Adsorption chromatography on aluminum oxide was usually applied to demonstrate the presence of progesterone in biological material such as adrenals from cattle (von Euw and Reichstein, 1941) or the corpus luteum of the whale (Prelog and Meister, 1949), or human placenta (Noall *et al.*, 1953 as well as Pearlman and Cerceo, 1952). Samuels (1947), and also Wiswell and Samuels (1951) developed a chromatographic procedure for the isolation of progesterone from liver-incubation mixtures. After development of the aluminum oxide column with hexane and hexane-chloroform (95:5 v/v), progesterone is eluted with hexane-chloroform (80:20 v/v). But this method proved to be inadequate for the isolation of the steroid from blood. Later Loy *et al.* (1957) used this procedure for the development of a routine method for the progesterone determination in luteal tissue. The authors supplemented chromatography with a countercurrent distribution of the progesterone fraction and achieved very satisfactory results. The column chromatography of Samuels (1947) was utilized also by Sommerville (1957) and Sommerville and Deshpande (1958) for routine determination of progesterone in plasma; chromatography is performed immediately after extraction of alkali-treated plasma (see Section

II, B). Without further purification efforts, the eluate containing progesterone was made to react with isonicotinic acid hydrazide and the formed derivative is quantified (see Section IV, C, 3). However, it seems somewhat doubtful if this purification procedure guarantees a sufficient specificity when only small amounts of progesterone are present.

Furthermore, column adsorption chromatography has been adopted as a routine method by Hinsberg *et al.* (1956). They chromatographed ether extracts of plasma on a column of 5 gm. aluminum oxide. After washing the column adequately with benzene, the progesterone fraction was eluted by a benzene-methylene chloride mixture (4:1v/v). One should not expect to obtain pure progesterone with this method and the authors themselves consider this procedure only a first purification, because it does not remove a significant amount of lipids, which interfere in subsequent paper chromatography using the systems of Bush. For this reason Hinsberg *et al.* (1956) transformed the progesterone in the eluate to its dinitrophenylhydrazine derivative according to Reich *et al.* (1952) (see Section IV, C, 2). Progesterone bisdinitrophenylhydrazone was separated from other reaction products by a subsequent chromatography on aluminum oxide. The progesterone derivative was eluted by benzene after the column was washed with a mixture of equal volumes of benzene and hexane.

Only Butt *et al.* (1951) have used column partition chromatography for the routine determination of progesterone. After thorough preliminary purification of the ethanol-ether (3:1 v/v) extracts of plasma with removal of the lipids, they used a column (6 mm. in diameter and 6 cm. long) of Hyflo Supercel using aqueous methanol as the stationary phase and *n*-hexane as the mobile phase. The conditions for this system have been investigated with great care and the most favorable ones for the separation of progesterone were found with the use of 70% methanol as the stationary phase. It is worthwhile to mention that this system permits a clear separation of progesterone from  $\Delta^4$ -androstene-3,17-dione which is only slightly more polar. Their partition coefficient (*K*) for progesterone is 1.05. Though only a few results are reported with the method of Butt *et al.* (1951) they agree well with the figures of authors who used paper chromatography for the isolation of progesterone. That Butt *et al.* (1951) could not find any progesterone in the peripheral venous blood under physiological conditions might be explained by the fact that not enough blood was used as starting material. The partition chromatographic system of Butt *et al.* (1951) was later used by Noall *et al.* (1953) for the successful isolation of progesterone from human placenta.

It seems that column chromatography in spite of some evident ad-

vantages, particularly for the quantitative spectrophotometric estimation, has not yet been fully utilized for routine progesterone determinations.

### C. PAPER CHROMATOGRAPHY

Paper chromatographic procedures have been successful when applied to microanalytical progesterone determinations. The great advantages lie in the simplicity, the remarkably sharp separation, and the possibility of an exact localization, even with very small amounts of progesterone.

Numerous systems suitable for the isolation of progesterone have been described. In selecting a method, several factors have to be considered, such as the extent to which the extracts are purified, the type of subsequent quantitative estimation, and last, but not least, local working conditions of a particular laboratory; also, the experience which the investigator or his team may have with one or more of the systems should be taken into account. For details the reader is referred to the reviews dealing exclusively with paper chromatography of steroids (Heftmann, 1955; Neher, 1958; Reineke, 1956). The present discussion is restricted to those systems which are of practical value for routine progesterone determinations.

Haskins *et al.* (1950) introduced a "monophasic" system (ethanol-water, 80:20) for the determination of progesterone in oily vehicles and placental tissue. But generally the monophasic solvent systems are not ideal for steroid chromatography. Chromatography on alumina paper has not been profitable, as shown by Edgar (1953a), especially for the isolation of progesterone from extracts of biological material.

Wide applications are known for systems with aqueous solvents as the stationary phase, e.g., the system A of Bush (1952) with 80% methanol as the stationary phase and light petroleum (b.p. 80–100°C.) as the mobile phase at 37°C. (Edgar, 1953a; Raeside and Turner, 1955) or variations of this system (Zander and Simmer, 1954; Hinsberg *et al.*, 1956; Short, 1958; Gorski *et al.*, 1958; Stormshak *et al.*, 1959). These systems, particularly the variations of Bush's system A, with lower alcohol concentration in the stationary phase, permit a clear separation of progesterone from other  $\Delta^4$ -3-ketosteroids as well as from impurities running with the solvent front. Recently we have obtained (Zander, 1960, unpublished results) very satisfactory results with the following system; 65% methanol : hexane at 37°C. The separation of progesterone ( $R_f$  value 0.69) from the solvent front was very good. It is not possible to separate  $\Delta^{11}$ -dehydroprogesterone, a compound closely related to progesterone, from the latter with any of the described systems. This compound, however, so far has not been demonstrated in biological material. Another sub-

stance, which might interfere is the "unknown  $\alpha,\beta$ -unsaturated ketone," which Hagopian *et al.* (1956) obtained in perfusates of human placentas. But this substance also was never encountered in the progesterone fractions from human placentae or from placental blood obtained with the above-named systems (Zander, 1956, unpublished results).

The Bush systems when used for the isolation of progesterone are effective only after a prepurification of the extracts. Even minute amounts of lipids may interfere during chromatography. On the other hand the great advantage derived from the Bush systems is that, after sufficient prepurification of the extracts and the paper, the eluted progesterone is obtained in a remarkable degree of purity. The ultraviolet spectrophotometry for the quantitative estimation can, as a rule, follow immediately after elution without further purification. Similarly, the eluates are suitable for infrared spectrophotometry.

Systems in which water is the only stationary phase are less suited for the purposes under discussion. Edgar (1953a) has tested such systems using light petroleum and aqueous propanol as the mobile phase. In his trials with ether extracts from blood to which progesterone had been added, he observed considerable tailing due to absorption.

Systems with nonaqueous solvents as the stationary phase, as proposed by Zaffaroni *et al.* (1950) and Burton *et al.* (1951), so far have not been generally accepted in the routine techniques for progesterone determinations in spite of their excellent separating qualities. This may be due to the difficulties encountered in removing the stationary phase from the paper before starting the quantitative assays. However, Wiest (1959) succeeded in quantitative assays of progesterone following the isolation by Zaffaroni chromatography. The method, measuring the absorption of incident ultraviolet light by the steroid directly on the paper chromatograms, compensated the effect of residual stationary phase by means of a similarly impregnated paper blank placed in front of the reference beam of the spectrophotometer. Savard's (1953) propylene glycol : methylcyclohexane system with an 8-hour running time has been used by Oertel *et al.* (1959). The  $R_{\text{Doc}}$  value (relative mobility referred to that of deoxycorticosterone) of progesterone lies between 4.8 and 5.2 in this system. Further purification of the paper eluate for the quantitative determination of progesterone is necessary (see Section IV, D).

Systems with less polar stationary phases (reversed phase systems) have not been applied for the practical determination of progesterone, although Edgar (1953a) found that a system with benzene as the stationary phase and 70% aqueous methanol as the mobile phase yielded very satisfactory results on silicone-treated paper ( $R_f$  value for progesterone in this system 0.68).

In general the important advantage of paper chromatography derives from the fact that, besides progesterone, other relatively weak polar  $\Delta^4$ -3-ketosteroids, which may be present in the extracts, can easily be recognized on the paper. Compounds like androst-4-ene-3,17-dione, testosterone, and 17 $\alpha$ -hydroxyprogesterone are clearly separated with most paper chromatographic techniques suitable for progesterone. These compounds may also be measured quantitatively. Such additional findings will be of biological interest.

In testing for progesterone it is important to detect other substances with progestational activity, such as the progesterone metabolites 20 $\alpha$ -hydroxypregn-4-en-3-one and its 20 $\beta$ -isomer, which have been isolated by Zander *et al.* (1958). Both these progestins are slightly more polar than progesterone and can easily be separated from the latter by paper chromatography of the extracts. They may also be quantified provided they are present in adequate amounts. For the differentiation and characterization of such progestational compounds, which accompany progesterone, it might be necessary to use a variety of solvent systems. In Tables III and IV are listed a series of systems which have been found useful for this purpose. Wiest (1956) has found that the 2 isomers

TABLE III

PAPER CHROMATOGRAPHIC BEHAVIOR OF 20 $\alpha$ -HYDROXYPREGN-4-EN-3-ONE AND ITS 20 $\beta$ -ISOMER IN COMPARISON TO PROGESTERONE AND OTHER WEAKLY POLAR STEROIDS IN DIFFERENT SOLVENT SYSTEMS<sup>a</sup>

Compound	Propylene glycol/ toluene (22°C.)	Formamide/ benzene- cyclohexane (1:1) (22°C.)	Bush B (22°C.)	Bush A (38°C.)
Deoxycorticosterone	1.00	1.00	1.00	1.00
20 $\alpha$ -Hydroxypregn-4-en-3-one	0.88	1.50	1.17	1.41
20 $\beta$ -Hydroxypregn-4-en-3-one	0.99	1.59	1.29	1.88
Progesterone	1.28	1.86	1.66	4.78
11-Ketoprogesterone	—	1.35	1.09	1.23
17 $\alpha$ -Hydroxyprogesterone	0.73	0.93	0.78	0.64
Testosterone	0.64	1.04	0.79	1.17
Epitestosterone	0.74	1.23	0.93	1.47
Androst-4-ene-3,17-dione	1.18	1.71	1.49	1.86
Adrenosterone	1.07	0.89	0.75	0.45
11 $\beta$ -Hydroxyandrost-4-ene-3,17-dione	0.60	0.32	0.32	—

<sup>a</sup> The values are related to deoxycorticosterone ( $R_{\text{DOC}}$  1.00). Data from Zander *et al.* (1958).

TABLE IV

PAPER CHROMATOGRAPHIC BEHAVIOR OF 20 $\alpha$ -HYDROXYPREGN-4-EN-3-ONE, 20 $\beta$ -HYDROXYPREGN-4-EN-3-ONE AND THEIR ACETATES IN COMPARISON TO PROGESTERONE AND ANDROST-4-ENE-3,17-DIONE

Compound	Phenyl-cellosolve/heptane <sup>a</sup>	Formamide/heptane <sup>a</sup>
Progesterone	1.00	1.00
Androst-4-ene-3,17-dione	0.67	0.29
20 $\alpha$ -Hydroxypregn-4-en-3-one	0.45	0.29
20 $\alpha$ -Hydroxypregn-4-en-3-one acetate	2.09	2.53
20 $\beta$ -Hydroxypregn-4-en-3-one	0.67	0.50
20 $\beta$ -Hydroxypregn-4-en-3-one acetate	2.21	2.80

<sup>a</sup> Values are related to progesterone ( $R_{\text{prog.}} = 1.00$ ). Data from Zander *et al.* (1958).

can be distinguished easily by the iodine color reaction. If the paper is treated with iodine the 20 $\alpha$ -isomer will give a blue color, whereas the 20 $\beta$ -isomer gives a brown color.

#### IV. Quantitative Estimation of Isolated Progesterone

##### A. SPECTROPHOTOMETRIC ESTIMATION AS $\Delta^4$ -3-KETOSTEROID

Progesterone as a compound with the  $\alpha,\beta$ -unsaturated ketone grouping exhibits a distinct ultraviolet absorption maximum at 240 m $\mu$  ( $\epsilon_{\text{max}} = 16,950$ ). The first workers to introduce a quantitative progesterone estimation method based on this maximal absorbancy were Reynolds and Ginsburg (1942), whose method has been adopted by numerous authors. If progesterone has been separated from other  $\Delta^4$ -3-ketosteroids with reasonable certainty before it is measured quantitatively, this method is reliable and satisfactory.

By using 1-cm-wide cells with a capacity of 3 to 3.5 ml., a minimum quantity of 2.5 to 5.0  $\mu\text{g.}$  progesterone can be measured with adequate accuracy. By the use of microcells the sensitivity of the method can be increased up to fivefold.

After the routine separation of progesterone from biological material the quantitative estimation yields, as a rule, a higher value for optical density than the one which would correspond to the quantity of progesterone actually present. The reason is the presence and interference of small quantities of nonspecific material which absorbs in the same ultraviolet region. The correction proposed by Allen (1950) eliminates such an error (see Section IV, E).



If an unusually large amount of nonspecific interfering material is contained in the final extracts, the absorption maximum may shift to shorter wavelengths. In such cases we recommend rechromatography of the material until an unequivocal maximum is obtained at 240 m $\mu$ .

To correct for absorbing material from the paper one should use an eluate of a piece of paper from a blank strip as a reference. The piece should be cut from a blank strip treated in the same fashion as the sample strip and the piece should correspond in size and location to that of the sample strip containing progesterone. Since most of the commercially available filter papers are contaminated with small quantities of material absorbing in the same region as progesterone, a thorough extraction of the paper before chromatography is necessary.

In order to judge the purity of the isolated material Zaffaroni and Burton (1951) have suggested a method which proved to be valuable. They proposed to compare the absorption curves of the isolated material and of a standard solution by computing all figures of measured optical density values as percentage of a maximum of 100. In the ideal case both curves must then coincide.

Quantitative estimation of progesterone directly on paper chromatogram strips has been used by Wiest (1959). This was done optically in a Cary recording spectrophotometer by measuring absorption of light as the chromatogram moved in front of a beam of wavelength 240 m $\mu$ . Background absorption was compensated for by means of a paper strip, similarly impregnated and dried, placed before the reference beam of the double-beam instrument. The method successfully measured 2  $\mu$ g. of chromatographed progesterone.

#### B. POLAROGRAPHIC ESTIMATION AS GIRARD COMPLEX

As Wolfe *et al.* (1940) have shown, certain ketonic steroids yield, after condensation with Girard's reagent T (trimethylacetylhydrazide ammonium chloride) under suitable conditions, well-defined polarographic waves. Quantitative polarographic estimations are possible, since the wave span is proportional to the steroid concentration. Amounts in the order of 10 $\mu$ g. can be measured with adequate accuracy.  $\alpha,\beta$ -Unsaturated ketosteroids are determinable with this method. They are characterized by a half-wave potential at about 1.1 volt.

On the basis of this principle, Butt *et al.* (1951) developed a method for the quantitative estimation of progesterone which is separated from purified extracts by partition chromatography as described in Section III, B. For the polarographic analysis of the Girard complex a technique was used which had been introduced by Barnett *et al.* (1946) for polaro-

graphic determination of 17-ketosteroids. The method of Butt *et al.* (1951) has not been used widely, since its sensitivity is not sufficient for the progesterone determination in plasma.

### C. SPECTROPHOTOMETRIC ESTIMATION OF PROGESTERONE DERIVATIVES

#### 1. Progesterone Bisthiosemicarbazone

The spectral characteristics of a series of thiosemicarbazide derivatives of  $\alpha,\beta$ -unsaturated ketones have been studied by Evans and Gillam (1943). They found the thiosemicarbazones useful for the identification of the  $\alpha,\beta$ -unsaturated carbonyl group, the maximum absorption band of which is displaced to about 300  $m\mu$  as compared with the 240  $m\mu$  of the free compound. This absorption range is suitable for quantitative work since nonspecific impurities absorb predominantly at shorter wavelengths. Another advantage is the very high intensity ( $\epsilon_{\max} = 29,950\text{--}37,000$ ) of

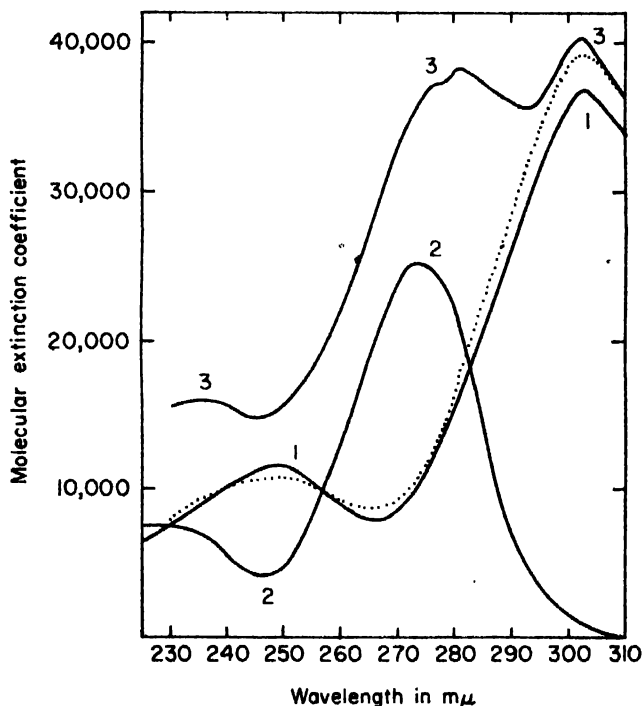


FIG. 1. Ultraviolet absorption spectra of the thiosemicarbazide derivatives of cholestenone (curve 1),  $3\beta$ -hydroxy- $5\alpha$ -pregnan-20-one (curve 2), and progesterone (curve 3) in absolute ethanol. The dotted curve, obtained by subtracting curve 2 from curve 3, represents the absorption due to the thiosemicarbazone of the  $\Delta^4$ -3-ketone group of progesterone. From Pearlman and Cerceo (1953).

the 300-m $\mu$  absorption band of the thiosemicarbazones. In addition, saturated carbonyl groups can be detected via their thiosemicarbazones which have an absorption maximum at 270–273 m $\mu$  ( $\epsilon_{\max} = 20,400\text{--}24,400$ ). However, it should be kept in mind that the application of the thiosemicarbazide method serves only to give an estimate of the total number of carbonyl groups whether or not they belong to a steroid molecule.

Figure 1 shows the ultraviolet absorption spectra of progesterone bis-thiosemicarbazone and of thiosemicarbazide derivatives of other steroids [molar extinction coefficients ( $\epsilon_{\max}$ ) at 280 m $\mu$ , 37,610 and at 301 m $\mu$ , 39,575]. This technique was first used for the determination of progesterone by Pearlman and Cerceo (1953) in their study of progesterone content in human placenta. The progesterone concentration in human placenta assessed by this method is in good agreement with values deriving from other physicochemical techniques.

Simple reaction conditions, for the preparation of thiosemicarbazide derivatives, originally introduced by Talbot *et al.* (1955) for the routine assay of urinary corticosteroids, have been used for supplementary identification and quantitative estimation of progesterone by Zander and von Münstermann (1956) as well as by Simmer and Simmer (1959) (see Section VII, A). According to our experience one may encounter difficulties with these procedures if progesterone is contaminated by trace amounts of ketonic impurities of the extracts or solvents.

### 2. Progesterone Bisdinitrophenylhydrazone

The 2,4-dinitrophenylhydrazine derivative of progesterone was prepared by Klein *et al.* (1948) and used for gravimetric determinations of this steroid in commercial solutions in oil. Reich *et al.* (1952) made use of the spectral characteristics of progesterone bisdinitrophenylhydrazone and arrived at a quantitative assay technique as well as a separation from pregnenolone. In a very careful investigation they studied the chromatographic and spectral behavior of the 2,4-dinitrophenylhydrazone derivatives of a large number of steroids (Reich *et al.*, 1953).

Progesterone bisdinitrophenylhydrazone has a yellow-reddish color, which can be easily recognized during chromatography with as little as 5  $\mu$ g. of material (see Section III, B).

The monodinitrophenylhydrazones of  $\Delta^4$ -3-ketosteroids exhibit an absorption maximum at approximately 290 m $\mu$  in chloroform. Saturated 3- or 20-ketosteroids which carry no hydroxyl group in the 17 or 21 position yield dinitrophenylhydrazone with an absorption maximum at 367–369 m $\mu$ . The bisdinitrophenylhydrazone of progesterone, having one carbonyl group in the  $\alpha,\beta$ -unsaturated position and one in the saturated side

chain, has an intense absorption maximum at  $380\text{ m}\mu$  ( $\epsilon_{\text{max}} = 36,665$  according to data given in 1956 by Hinsberg *et al.*).

Since unreacted 2,4-dinitrophenylhydrazine shows a maximum at  $342\text{ m}\mu$  it is necessary to remove the excess dinitrophenylhydrazine prior to the quantitative determination. According to the work of Reich *et al.* (1952) this is possible by oxidizing it with either Fehling's solution or Benedict's reagent. The reaction product is *m*-dinitrobenzene. Separation of progesterone bisdinitrophenylhydrazone from other reaction products may be effected by chromatography (see Section III, B). The method of Reich *et al.* has been adapted to microscale work by Hinsberg *et al.* (1956) who utilized this procedure after preliminary purification of the extracts (see Section III, B) to determine progesterone quantitatively in human plasma. It should be mentioned, however, that the purification proposed by Hinsberg *et al.* (1956) is feasible only if the plasma does not contain more than a minimal amount of lipids. With blood as a starting material the results with this method are not satisfactory (Ott and Pelzer, 1958) because some nonspecific impurities in the ether-soluble fraction of blood behave like progesterone during the aluminum oxide chromatography of the ether extracts and during chromatography of the 2,4-dinitrophenylhydrazine derivatives. With a maximal absorbancy at  $366\text{ m}\mu$  these 2,4-dinitrophenylhydrazine derivatives may prove to be a very disturbing factor in the progesterone determination.

### 3. Progesterone Bisisonicotinic Acid Hydrazone

Isonicotyl hydrazones of several ketosteroids have been prepared by Ercoli *et al.* (1952). Like the 2,4-dinitrophenylhydrazine derivative the bisisonicotinic acid hydrazone of progesterone also shows a maximal absorption at  $380\text{ m}\mu$ . The reaction is relatively insensitive. According to Short (1961) the molar extinction coefficient ( $\epsilon_{\text{max}}$ ) is 11,800. It has been used by Umberger (1955) for the quantitative determination of relatively large amounts of progesterone in oily solution. Weichselbaum and Margraf (1957) utilized the isonicotinic acid hydrazine as a reagent for the determination of  $\Delta^4$ -3-ketocorticosteroids in human plasma. The same reaction has been adapted by Sommerville and Deshpande (1958; Sommerville, 1957) to a microscale for the quantitative determination of progesterone in preliminary purified extracts of human plasma (see Section III, B). The quantitative estimation is performed without further purification of the resulting isonicotinic acid hydrazone, by applying the Allen correction formula. It has been mentioned above (Section III, B) that it seems somewhat doubtful whether the purification procedure before the quantitative measurement guarantees a sufficient specificity when only small amounts of progesterone are present.

## D. SULFURIC ACID-ETHANOL REACTION

Utilizing the findings of Zaffaroni (1950) and Axelrod (1953) who showed that the absorption spectrum of progesterone in concentrated sulfuric acid exhibits a distinct maximum at 290  $m\mu$ , Oertel *et al.* (1959) modified the method of Zander and Simmer (1954) employing 66% sulfuric acid in 80% ethanol for the quantitative estimation of progesterone. The sulfuric acid concentration was decreased to 66%, because it was observed that the concentrated sulfuric acid reacts with contaminating substances, present in biological material, to produce ultraviolet absorption which interferes with that of progesterone. According to Oertel *et al.* (1959) the use of a lower sulfuric acid concentration eliminates the appearance of undesirable background interference.

This reaction is not very specific but it is slightly more sensitive than the spectrophotometric estimation of progesterone in ethanol at 240  $m\mu$ . According to Short (1961) the molar extinction coefficient ( $\epsilon_{\max}$ ) is approximately 19,000. An adequate separation of progesterone from other steroids before the treatment with sulfuric acid is necessary. The method is described in detail in Section VII, C.

One disadvantage of the method of Oertel *et al.* is that the sulfuric acid treatment makes it impossible to identify the isolated material with progesterone, a most valuable protective measure when the slightest doubt prevails.

## E. FLUORESCENCE REACTIONS

Abelson and Bondy (1955) have described an alkali fluorescence reaction for the estimation of  $\alpha,\beta$ -unsaturated ketosteroids. The reagent consists of a 0.1–0.3 *N* solution of potassium tert-butoxide, freshly prepared by refluxing molten potassium metal with tertiary butanol. It is not possible to estimate concentrations of less than 1  $\mu g$ . progesterone per milliliter solvent with any degree of accuracy by this method (Short, 1961). Therefore this fluorescence reaction for progesterone estimation is less sensitive than other available methods.

Touchstone and Murawec (1960) recently described a new fluorometric method consisting of heating progesterone at 60° for 30 minutes in 2 *N* potassium hydroxide in methanol before dissolving it in sulfuric acid at room temperature. The reaction caused the appearance of a new peak at 380  $m\mu$  in the absorption spectrum of progesterone in sulfuric acid. Fluorescence was maximal at 490  $m\mu$  when excitation was carried out at 390  $m\mu$ . According to Touchstone and Murawec this method results

in a hundredfold increase of fluorescence over that found with sulfuric acid alone.

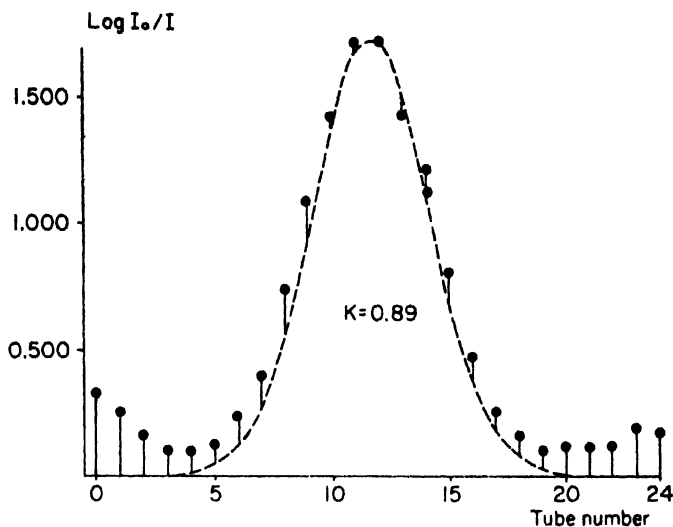
#### F. APPLICATION OF THE COLOR CORRECTION EQUATION OF ALLEN

In most of the presently available methods for the quantitative estimation of progesterone in plasma and tissues the correction formula of Allen (1950) is applied, because of the almost inevitable presence of accompanying nonsteroidal material, which interferes in the range of maximal spectral absorption of progesterone. The correction formula serves to eliminate these interferences by a simple computation, so that the adjusted value indicates the actual quantity of progesterone or its derivatives.

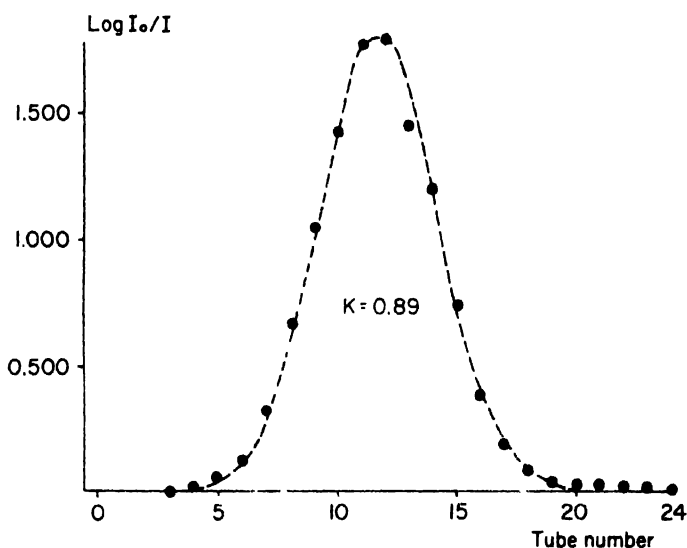
Allen's formula is based on the assumption that the absorption curve of the interfering substances approaches closely linearity in the region of the absorption maximum of the steroid to be measured. If this condition is not fulfilled the formula may yield erroneous estimates.

Several authors have tried to ascertain experimentally whether this assumption is valid in a given case. Loy *et al.* (1957) used the fractions preceding and following the progesterone fraction in their column chromatographic procedure for the purification of progesterone (see Section III, B). They found that the absorption curves of the two discarded fractions from the column approach linearity in the region in which progesterone exhibits its absorption maximum. This of course is only suggestive, since the impurities of the two discarded fractions do not necessarily have the same absorption spectra as those in the progesterone fraction. Short (1958) extracted and purified ox plasma, with no progesterone added, by his method, and the area of the chromatogram corresponding in  $R_f$  value to progesterone was cut out, eluted, and measured in the spectrophotometer. He found linear absorption in the region where progesterone gives its maximum absorption. Oertel *et al.* (1959) using their own method arrived at the same result with human male plasma. But it seems somewhat doubtful whether the results of these experiments can be accepted in general as a test for the validity of Allen's correction for all cases, because it may well be that other materials, e.g., human female plasma, contain different interfering substances in the progesterone fraction.

Probably the best experimental approach to the problem has been proposed by Diczfalussy (1955). His method has the advantage of being applicable also with material for which no comparative, steroid-free matter is available, as is the case with placenta or corpus luteum tissue. The principle of the method rests upon the separation of the interfering mate-



(A)



(B)

FIG. 2A. Countercurrent distribution of a pool of progesterone isolated by paper chromatography from full-term placentae with the method of Zander and Simmer (1954). In upper phase: *n*-hexane. In lower phase: 49% (v/v) ethanol. Filled circles: uncorrected optical densities at 240  $m\mu$ . Dotted line: theoretical distribution values. Identity of the distributed material with progesterone was established on infrared spectrographic analysis of the contents of tubes 9 to 16.

FIG. 2B. Same data following the application of Allen's correction equation (with readings at 225, 240, and 255  $m\mu$ ). In tubes 0 to 3 negative values were obtained following correction. From Diczfalussy (1955) and Zander and von Münstermann (1956).

rial from pooled steroid fractions of a certain starting material by countercurrent distribution. The absorption curve of the interfering material can then be examined.

The following gives an example for the actual performance of the method. Diszfalusy tested the applicability of Allen's formula with a pool of progesterone, isolated from human placenta, by the method of Zander and Simmer (1954; Zander and von Münstermann, 1956). Figure 2A shows the results of the distribution with optical density measurement at only 240  $m\mu$ . The partition coefficient ( $K = 0.89$ ) is in close agreement with that of progesterone. Identity of the contents of tubes 9 to 16 with progesterone was established by infrared spectrophotometric analysis. Application of Allen's correction with readings at 225, 240, and 255  $m\mu$  is given in Fig. 2B. It shows close agreement between theoretical distribution and corrected optical densities. Therefore one would expect linear absorption of the interfering material in the region from 225 to 255  $m\mu$ . The absorption of the combined contents of tube 0 to 3 and 21 to 24 with most of the interfering substances is shown in comparison to the absorption curve of authentic progesterone in Fig. 3. It is apparent

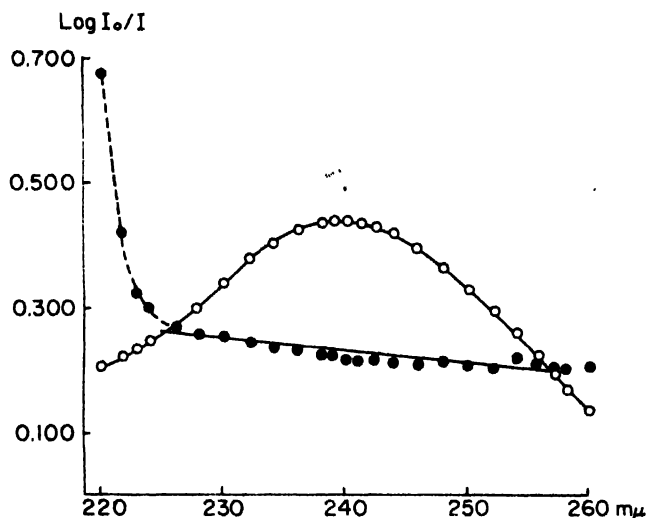


FIG. 3. Ultraviolet absorption spectra of crystalline progesterone (open circles) and of the interfering material collected from tubes 0 to 3 and 21 to 24 of the distribution shown in Figs. 2A and B (filled circles). From Diczfalusy (1955) and Zander and von Münstermann (1956).

from this figure that the absorption of the impurities fulfills closely the condition of linearity. Thus the application of Allen's formula is justified and it will improve the precision of estimation when the above-mentioned method is used for the determination of progesterone in placental tissue.



It should be mentioned here that it is also possible to test the validity of the Allen correction in a given case without chemical fractionation of the extract. O'Sullivan (1958) has described an extension to the Allen color-correction procedure which is suitable for routine application and which uses the shape of the absorption curves of the pure compound and of the extract to check the linearity of the background absorption.

## V. Additional Identification of Progesterone

To be certain that the findings obtained in employing routine methods for the determination of progesterone represent the hormone concentrations, additional identification of the material is advisable. This is particularly necessary when no data on the progesterone content of the matter under investigation are available. Such supplemental identification of the isolated material is also recommended if the progesterone concentration in plasma is to be established under conditions (physiological or pathological) which have not been hitherto studied. This is the only way to avoid errors and thus as complete an identification as possible should be considered an indispensable requirement for unequivocal evidence. Techniques, which do not permit a recovery of the isolated material after their quantitative estimation have the great disadvantage of making a supplementary identification impossible.

In this section only chemical methods are described which provide a supplementary identification. It is not necessary to emphasize that biological assays may also serve to identify the isolated material.

### A. COLOR REACTIONS

Additional information about the nature of an isolated substance might be provided by color reactions particularly when they are applied on the filter paper following paper chromatography. Although color reactions are in many cases nonspecific, they are nevertheless of practical value to indicate the position of progesterone on the paper.

From the great number of known color reactions only a few, which proved to be valuable for progesterone, can be described within the limits of this chapter. The reader is referred to Neher (1958), who gives an extensive review of the literature. The details of the methods can be obtained from the original reports.

*Phosphomolybdic acid (5% alcohol solution) (Kritchevsky and Kirk,*

1952). This very simple test yields a blue color on yellow-greenish background on the filter paper after heating to 90°C. for 5 to 10 minutes. The reaction is relatively sensitive for progesterone but also nonspecific; 2.0  $\mu$ g. on 1-cm<sup>2</sup>. paper can be detected.

*Sodium hydroxide fluorescence* (Bush, 1952). This reaction is relatively specific for all  $\Delta^4$ -3-ketosteroids. By using 10% NaOH in 60% methanol and heating 15 minutes to 70°–100°C. 0.5  $\mu$ g. progesterone on 1-cm<sup>2</sup>. paper can be recognized. To increase specificity this practical method may be combined with the demonstration or exclusion of reducing steroids, by tetrazolium salts (e.g., blue tetrazolium, Simpson *et al.*, 1954; Ayres *et al.*, 1957).

*2,4-Dinitrophenylhydrazine* (Reich *et al.*, 1950). This reaction is specific for reactive keto groups (see Section IV, C, 2) and especially sensitive for  $\Delta^4$ -3-ketones. It may be performed directly on the paper, where it yields a yellow-orange color in the presence of progesterone (Kochakian and Stidworthy, 1952; Reineke, 1956).

*Antimony trichloride* (Neher and Wettstein, 1951). Though this reaction is rather nonspecific it happens to be extremely sensitive for progesterone, particularly when used in the modification of Shull *et al.* (1952). After pretreatment of the paper for 20 minutes with chlorine gas, a saturated antimony trichloride solution in acetic anhydride is sprayed on the paper which is then exposed for 4 minutes to a temperature of 90°C. In this way 0.25  $\mu$ g. of progesterone on 1-cm<sup>2</sup>. paper may be detected. In the visible light range a yellow color is seen, and yellow-orange fluorescence develops with ultraviolet light.

## B. SULFURIC ACID REACTION

A valuable method which has been repeatedly employed to ascertain the identity of the isolated material with progesterone is to determine the absorption spectrum in concentrated sulfuric acid according to Zaffaroni (1950). It is rather characteristic for  $\Delta^4$ -3-ketosteroids which lack hydroxyl groups to exhibit a distinct absorption maximum at 290  $m\mu$ . The technique is simple. The isolated material is evaporated to dryness and dissolved in concentrated sulfuric acid. The reaction product is permitted to stand for 2 hours at room temperature and the absorption is determined between 220 and 600  $m\mu$ . In the presence of  $\Delta^4$ -3-ketosteroids carrying hydroxyl groups other absorption maxima besides the characteristic one at 290  $m\mu$  are usually observed. Zaffaroni's technique has been modified by Oertel *et al.* (1959) resulting in a very sensitive method for the quantitative determination of progesterone (see Sections IV, D and

VII, C). This modification requires a solution of approximately 66% sulfuric acid in ethanol instead of the concentrated sulfuric acid.

### C. FORMATION OF DERIVATIVES

A helpful method to confirm the identity of the isolated material consists in the formation of progesterone derivatives and the determination of their characteristics. Only a few are mentioned here, particularly those suitable for microquantities.

In case of a diketone, like progesterone, the ketonic reagents should be considered a first choice. Of these thiosemicarbazide (Pearlman and Cerceo, 1953; Zander and von Münstermann, 1956; Simmer and Simmer, 1959), 2,4-dinitrophenylhydrazine (Reich *et al.*, 1952; Hinsberg *et al.*, 1956), and isonicotinic acid hydrazine (Umberger, 1955; Sommerville and Deshpande, 1958) have proven to be of practical value. The corresponding derivatives of these reagents exhibit characteristic shifts of the ultraviolet absorption maximum and may also be a serviceable tool for quantitative measurements. The reactions are described in more detail in Section IV, C, 1-3. It is obvious that the derivatives formed in this fashion may also be examined for their mobility in column or paper chromatography.

To exclude the presence of reactive hydroxyl groups it may be worthwhile to try acetylation with acetic anhydride in pyridine. If the isolated material consists only of progesterone its mobility in a second chromatogram should not change.

The reduction of the C-20 keto group to the hydroxyl group offers further possibilities to form derivatives, particularly by enzymatic reactions when only microquantities are available. According to Wiest (1960) 20 $\alpha$ -hydroxypregn-4-en-3-one is formed in the presence of 20 $\alpha$ -hydroxy steroid dehydrogenase obtained from rat ovaries. Similarly, according to our own experiences (Zander, 1960, unpublished data), one can obtain 20 $\beta$ -hydroxypregn-4-en-3-one by using crystallized 20 $\beta$ -hydroxy steroid dehydrogenase isolated by Hübener *et al.* (1959) from *Streptomyces hydrogenans*. Both isomers can be acetylated and the free as well as the acetylated compounds can be recognized by their mobility in column or paper chromatography.

### D. INFRARED ABSORPTION SPECTRUM

For a conclusive identification it is most desirable to compare the infrared spectrum of the isolated material with the spectrum of authentic

progesterone. Thanks to the potassium bromide preparation method, introduced by Schiedt and Reinwein (1952) and by Stimson and O'Donnel (1952), such a comparison has become feasible even if only very minute quantities of progesterone can be isolated, though occasionally it may be necessary to accumulate material derived from several isolations until the amount is adequate. For details the reader is referred to the original literature. If the material has been isolated by paper chromatography it is necessary to prepare an eluate of the blank filter paper and to expose this eluate to infrared spectrophotometry for comparative purposes.

## VI. Reliability of Different Methods

As pointed out by Borth (1952, 1957) the essential characteristics for the reliability of hormone assay methods are specificity, accuracy, precision, and sensitivity. How far these four criteria are fulfilled by the presently available methods to determine progesterone in blood or tissue will be discussed in the following paragraphs. Those experimental data published in the literature, which permit a critical judgment of one or more of these criteria with respect to a single procedure are summarized in Tables VI and VII.

### A. SPECIFICITY

Specificity means that the method of analysis should measure progesterone only and nothing else. Conclusions as to the specificity may be drawn first from the principles on which a method is based and from the various steps applied in such method. This has been discussed in Sections II to V. In general it can be stated that an adequate specificity of microanalytical procedure can only be expected if satisfactory chromatographic separation methods for the separation of progesterone from sufficiently purified extracts are used. Nevertheless with all routine methods so far described one obtains only a more or less tentative identification of the isolated substance presumed to be progesterone. It is therefore most desirable to check the specificity of the method by supplementary examination of the isolated material. A number of physicochemical techniques have been recommended for such supplementary identification of microquantities. They have been discussed in Section V.

Table V lists the various procedures applied for the supplementary identification of material obtained with the variety of routine determination methods and allows conclusions as to the evidence for the specificity of their results.

TABLE V  
METHODS APPLIED FOR ADDITIONAL CHARACTERIZATION  
OF MATERIAL ISOLATED AS PROGESTERONE

Method <sup>a</sup>	Material	Additional characterization
Butt <i>et al.</i> (1951)	Plasma	
Edgar (1953a)	Blood, follicle fluid, luteal tissue <sup>b</sup>	Mobility in reversed phase paper chromatography, NaOH fluorescence, <i>m</i> -dinitrobenzene color reaction
Zander and Sinmer (1954)	Plasma, <sup>c</sup> follicle fluid, luteal tissue, <sup>d</sup> placental tissue, <sup>e</sup> fat tissue <sup>f</sup>	Infrared absorption spectrum, formation of thiosemicarbazone, sulfuric acid chromogen, NaOH fluorescence
Haskins (1954)	Placental tissue	Biologic assay
Raesside and Turner (1955)	Plasma	Dinitrophenylhydrazine and <i>m</i> -dinitrobenzene color reactions
Hinsberg <i>et al.</i> (1956)	Plasma	---
Loy <i>et al.</i> (1957)	Luteal tissue	Biologic assay, mobility in paper chromatography
Short (1958)	Plasma <sup>g,h</sup>	Infrared absorption spectrum, sulfuric acid chromogen, <i>m</i> -dinitrobenzene reaction, NaOH fluorescence
Sommerville and Deshpande (1958)	Plasma	UV absorption at 240 m $\mu$ , mobility in paper chromatography
Gorski <i>et al.</i> (1958)	Luteal and ovarian tissue	Sulfuric acid chromogen
Oertel <i>et al.</i> (1959)	Plasma	

<sup>a</sup> For papers containing additional information on the identification of isolated material, see the footnotes.

<sup>b</sup> Edgar (1953b).

<sup>c</sup> Zander (1954, 1955).

<sup>d</sup> Zander (1954); Zander *et al.* (1958).

<sup>e</sup> Zander and von Münstermann (1956).

<sup>f</sup> Kaufmann and Zander (1956).

<sup>g</sup> Short and Eton (1959).

<sup>h</sup> Balfour *et al.* (1957). Variant from the method of Short (1958), an additional 9-tube countercurrent distribution, for preliminary purification has been used.

## B. ACCURACY

The accuracy of a measurement signifies, according to Borth's definition, the closeness with which it approaches the "true" value. It can be determined by the percentage recovery of pure authentic compounds added to samples before analysis. Table VI demonstrates that 70—

TABLE VI  
ACCURACY, PRECISION, AND SENSITIVITY OF DIFFERENT ROUTINE METHODS

Method	Accuracy		Precision	Sensitivity <sup>b</sup> (smallest amount of pure progesterone that can be measured; $\mu\text{g.}$ )
	Progesterone added to the sample before analysis	Recovery <sup>a</sup> (% $\pm$ S.D.)		
Butt <i>et al.</i> (1951)	10-30 $\mu\text{g.}$ to 20 ml. human plasma	90 $\pm$ 3.5 (9)	---	1.0
Edgar (1953a)	4-60 $\mu\text{g.}$ to 40 ml. human blood	74.2 $\pm$ 4.0 (5)	---	2.5-5.0
Zander <i>et al.</i> (method described in this chapter)	11.7-22.0 $\mu\text{g.}$ to 20 gm. placental mash	90 $\pm$ 2.5 (7)	S.D.(s) of 4 duplicate determinations with 2.2-11.4 $\mu\text{g.}$ added to 30-49 ml. human plasma = $\pm$ 0.62 $\mu\text{g.}$ [ $\pm$ 0.60 $\mu\text{g.}$ ] <sup>d</sup> S.D.(s) of 4 duplicate determinations with 0.5-1.32 $\mu\text{g.}$ added to 40-49 ml. human plasma = $\pm$ 0.63 $\mu\text{g.}$ [ $\pm$ 0.97 $\mu\text{g.}$ ] <sup>d</sup>	0.5-1.0 <sup>c</sup>
	2.2-11.4 $\mu\text{g.}$ to 30-49 ml. human plasma	81 $\pm$ 4.8 (8)		
	0.5-1.32 $\mu\text{g.}$ to 40-49 ml. human plasma	97 $\pm$ 3.1 <sup>d</sup> 71 $\pm$ 24.0 (8)		
Haskins (1954)	1 mg. to 140-260 gm. placental mash	76.3 $\pm$ 8.1 (5)	---	2.5-5.0
Raesside and Turner (1955)	10-40 $\mu\text{g.}$ to 20 ml. plasma	70-80 <sup>c</sup>	---	2.5-5.0
Hinsberg <i>et al.</i> (1956)	No data for calculation available	---	---	0.5
Loy <i>et al.</i> (1957)	No data on the amount added to luteal tissue	77-92 <sup>c</sup>	S.D.(s) of 7 duplicate determinations on luteal tissue = $\pm$ 4.9 $\mu\text{g.}$ (range 25.4-213 $\mu\text{g.}$ )	2.5-5.0



90% recoveries are claimed for the routine determination methods of progesterone.

To evaluate comparatively the recoveries by various methods, however, one has to consider the amounts of added progesterone as well as the quantities of plasma or tissue used as the starting material. A change in one of the two factors may possibly also change the percentage recovery. Thus a comparison of the recovery data given in Table VI can be made only with some reservation. Nevertheless the percentage recovery of added progesterone, within the range of the amounts tested, is quite satisfactory in all the described methods. In general, as far as accuracy is concerned, those methods are preferred which yield good percentage recoveries of very small amounts of progesterone added to relatively large amounts of plasma or tissue.

Accuracy can be best judged by adding radioactive progesterone to the test sample in a routine fashion, as shown in Section VI for the methods of Oertel *et al.* as well as of Zander *et al.* The final value for progesterone, as calculated from the loss of radioactivity, naturally approaches the "true" value of a single determination very closely.

### C. PRECISION

The term precision is concerned merely with the reproducibility of the measurements. Precision may be judged by the standard deviation of results obtained from replicate determinations of the same sample. The experimental data which permit an estimate of the precision of the various methods are assembled in Table VII. To judge these data critically one again has to consider the amount of progesterone as well as the quantity of plasma or tissue. Precision is completely defined only when quantities have been tested which approach the lower end of the sensitivity scale. In this respect the available data for the different methods are still incomplete.

### 1). SENSITIVITY

(One way to ascertain the sensitivity of a method, is to estimate the smallest amount of added progesterone that can be recovered with adequate accuracy and precision. Sensitivity can be also calculated from the standard deviation of replicates. The data published up to the present time for both values are listed in Table VI. In several instances, no recovery or replicate experiments have been reported for quantities close to the lower limits of sensitivity of the methods. Thus an exact evaluation of the sensitivity is not possible in these cases. The values in the



column "sensitivity" of Table VI represent only the smallest amount of pure progesterone that can be measured by the procedure for the quantitative assay; however, as indicated in the discussion on the precision, it is an open question whether or not the precision attainable at this level is adequate.

## VII. Description of Recommended Methods<sup>1</sup>

### A. METHOD OF ZANDER AND SIMMER (1954)<sup>2</sup>

*Reagents.* All reagents are of analytical grade; solvents should be redistilled before use.

<sup>1</sup> The available data for the reliability of the recommended methods are included for the specificity in Table V and for accuracy, precision, and sensitivity in Table VI.

At the suggestion of Dr. L. T. Samuels the three methods recommended here were compared. Five "unknown" samples of 22 ml. human plasma containing varying amounts of added progesterone were analyzed in each of the three laboratories responsible for these methods. The results were recently published by Short (1961) and are summarized in the following table:

Sample No.	Progesterone in "unknown" sample (μg./100 ml.)	Zander (μg./100 ml.)	Short (μg./100 ml.)	Oertel and Eik-Nes (μg./100 ml.)
1	8.1	8.11	8.6	7.2 7.4
2	5.0	3.80	5.0	5.0 3.6
3	Blank	0	1.0	1.2
4	25.0	25.55	28.0	25.0 23.7
5	25.0	25.55	26.9	25.2 24.3

All results in the table have been corrected for losses. Zander submitted the extracts of the samples twice to chromatography. No radioactive progesterone, as described in this paper for the method of Zander and Simmer, was added. An over-all mean recovery of 70% for each chromatography with a total amount of less than 5.0 μg. progesterone and of 75% with a total amount of more than 5.0 μg. was assumed. Short assumed an over-all mean recovery rate of 73% and corrected all results by a factor of 1.37. Oertel and Eik-Nes added progesterone-4-C<sup>14</sup> (2000 c.p.m.) to each sample before extraction, the samples being run in duplicate. They obtained recoveries for sample 1, 74%, 74%; for sample 2, 74%, 58%; for sample 3, 75%; for sample 4, 74%, 66%; for sample 5, 80%, 82%.

<sup>2</sup> The description of the method corresponds to the procedure now used in our Laboratory. It takes into consideration the modification of Zander and von Münstermann (1956) as well as some unpublished results.

*Precipitation of protein from plasma.* Up to 200 ml. of blood is collected into a vessel containing an anticoagulant (sodium oxalate, sodium citrate, or heparin). After standing for 1 to 2 hours at  $+2^{\circ}$  to  $+4^{\circ}\text{C}$ . the sample is centrifuged for 20 minutes. The plasma is separated and 1000 c.p.m. progesterone-4- $\text{C}^{14}$  of high specific activity are added. With mechanical stirring, the plasma is slowly (5 ml. in 3 minutes) added to 5 volumes of ethanol-ether (3:1 v/v). The mixture is then centrifuged, the supernatant decanted, and the remaining precipitate is extracted twice with half the volume of the ethanol-ether each time. All ethanol-ether extracts are combined.

*Precipitation of protein from tissue.* Up to 40 gm. of tissue are cut into small pieces immediately after dissection and freed from blood on filter paper. The tissue pieces are weighed and homogenized. A fivefold quantity of an ethanol-ether (3:1 v/v) mixture containing 1000 c.p.m. progesterone-4- $\text{C}^{14}$  is added to the homogenate while still in the homogenizer, and the homogenization is continued for another 5 minutes. After centrifugation the ethanol-ether extract is decanted and the precipitate washed with half the volume of the ethanol-ether each time. All the extracts are combined.

*Extraction with ethyl acetate.* The combined ethanol-ether extracts from plasma or tissue are concentrated *in vacuo* at  $40^{\circ}\text{C}$ . to a volume of 2 to 3 ml., which is then diluted with 40 ml. of distilled water. This aqueous extract is transferred to a separatory funnel and extracted 3 times with 60 ml. of ethyl acetate each time. The combined ethyl acetate extracts are dried over sodium sulfate and evaporated to dryness *in vacuo* at  $40^{\circ}\text{C}$ .

*Removal of lipid material.* In a water bath ( $40^{\circ}\text{C}$ .) the residue of the ethyl acetate extract is taken up 5 times in 2 ml. of 70% methanol and transferred into a centrifuge vessel. The dissolution of the residue requires some time. Occasionally the residue is not completely soluble, but the insoluble material does not contain progesterone. The combined aqueous methanol extract is kept in a deep freeze at  $-15^{\circ}\text{C}$ . for 18 hours during which time the lipids precipitate almost quantitatively. To complete the precipitation the vessel is centrifuged in a refrigerated centrifuge for 15 minutes at  $-15^{\circ}$  to  $-20^{\circ}\text{C}$ . Although a satisfactory precipitation may be obtained at a speed of 3000 r.p.m., the best results are attained using a speed of 15,000 r.p.m. Immediately after centrifugation the supernatant is transferred to a 100-ml. separatory funnel, diluted with 20 ml. of water, and extracted 3 times with 20 ml. of petroleum ether ( $35-40^{\circ}\text{C}$ .). The combined light petroleum extracts are evaporated to dryness *in vacuo*.

*Paper chromatography.* The dried residue of the petroleum-ether

extract is transferred to a 1.5-ml. cone-shaped microflask using methanol which is then evaporated under a nitrogen stream. The residue is applied on a 3-cm.-wide paper strip (Schleicher and Schüll, No. 2045 b. or Whatman No. 1) with a micropipet using 0.1 ml. of methanol, 0.1 ml. of chloroform twice, and then 0.1 ml. of methanol again. On a second paper strip 10  $\mu$ g. of authentic progesterone are placed as a reference compound. A third strip is used as the paper blank. All paper strips must be prewashed for at least 48 hours with absolute methanol at 37°C.

The paper strips are transferred to a tank for paper chromatography containing 80% methanol as the stationary phase and ligroin as the mobile phase or 65% methanol as the stationary phase and hexane as the mobile phase. Descending paper chromatography is used for 4 hours at 37°C., after 3 hours equilibration at the same temperature. The latter system has the advantage that progesterone with its relatively low  $R_f$  value (0.69) is better separated from the impurities which migrate with the solvent front.

*Elution of progesterone.* The progesterone spot on the paper is located by ultraviolet (UV) contact photography. Should the progesterone quantity be so small that it cannot be detected satisfactorily by the UV contact photography, the locus of the progesterone-4- $C^{14}$  on the paper strip used for the unknown material can be traced by a gas flow counter. The area containing progesterone is eluted for 30 minutes with 2–5 ml. of optically pure absolute methanol using the elution apparatus shown in Fig. 4. A piece of paper from the blank strip corresponding in size and location to that of the sample strip is eluted in the same fashion with the same amount of methanol.

*Quantitative estimation.* One-tenth of the eluate from the sample strip is plated and counted in a gas flow counter. The absorption of the remaining methanol solution, using the paper blank as a reference, is measured in an UV spectrophotometer between 220 and 270  $m\mu$  in intervals of 5  $m\mu$ . In the region of maximal absorption the intervals are reduced to 1  $m\mu$ . Should the maximal absorption not be found at 240  $m\mu$  rechromatography is recommended. The maximum absorbance at 240  $m\mu$  is corrected by the Allen formula:

$$\text{O.D.}_{240 \text{ corr}} = \text{O.D.}_{240} - \frac{\text{O.D.}_{225} + \text{O.D.}_{255}}{2}$$

The amount of progesterone present in the sample is finally calculated from the absorbancy of a progesterone standard. Losses are calculated from the recovery of the radioactive progesterone added to the plasma (see above).

With less than 3–5  $\mu\text{g.}$  of progesterone in the eluate of the sample, microcells have to be used for the measurement.

*Formation of progesterone bithiosemicarbazone.* This step has shown its value as a routine supplementary characterization of the isolated ma-

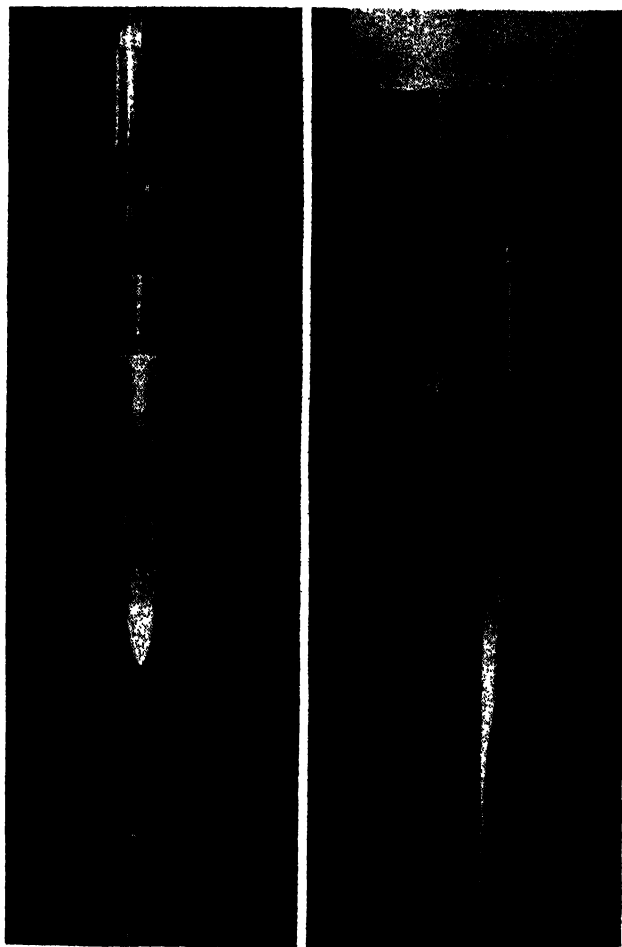


FIG. 4. Apparatus for standardized elution of progesterone or other steroids from paper strips

terial and as an alternative method for the quantitative determination of progesterone (see Section IV, C, 1).

To the dried eluates of the strips with the sample and the blank one adds 0.5 ml. of a 0.1 *M* thiosemicarbazide solution in 0.1 *N* hydrochloric acid, and 3 ml. of absolute methanol, which has been distilled over thiosemicarbazide. The mixture is permitted to stand at room temperature for 90 minutes. The absorption of the mixture with the un-

known sample is then measured against the mixture with a blank in an UV spectrophotometer. If progesterone is present, the UV absorption maximum will now be found at 301–302 m $\mu$ . The quantity of progesterone present in the sample can be determined from the absorbancy of a progesterone bithiosemicarbazone standard.

### B. METHOD OF SHORT (1958)<sup>3</sup>

*Reagents.* All solvents are redistilled before use. The light petroleum is the 40°–60°C. b.p. fraction analytical grade.

*Extraction procedure.* Blood is collected into a vessel containing an anticoagulant (sodium oxalate, sodium citrate, or heparin). The samples are kept in a refrigerator at about +5°C. and the plasma is separated by centrifugation a few hours later. The plasma is taken off and a 5 *N* NaOH solution is added to give a final concentration of 0.5% NaOH in the plasma. After thorough stirring, the plasma is transferred to a separatory funnel and extracted 6 times with a quantity of ether corresponding to 4 times the volume of the plasma. The combined ether extracts are washed once with a tenth of their volume of distilled water and evaporated to dryness on a warm water bath, the final traces of solvent being blown off under a stream of nitrogen. Occasionally it may happen that samples tend to gel after the addition of sodium hydroxide to the plasma; however, this does not appear to interfere with the assay.

*Partition between light petroleum and 70% methanol.* The residue is redissolved, using 3 successive portions of 10 ml. of light petroleum (b.p. 40°–60°C.). This solution is extracted 6 times with 10-ml. portions of 70% methanol in a separatory funnel. The combined methanolic extracts are then evaporated to dryness in a 500-ml. round-bottomed flask, care being taken not to heat the flask above 70°C. The residue is then transferred to a 2.5-ml. glass test tube with washings of 1.1 and 0.5 ml. absolute methanol, and this solution is subsequently evaporated to dryness under a stream of nitrogen in a warm water bath.

*Paper chromatography, elution of progesterone, and quantitative estimation.* The residue is redissolved in 0.05 ml. of absolute methanol and transferred to a sheet of Whatman No. 20 chromatography paper, including two additional washings each of 0.025 ml. methanol. The paper chromatography, elution, and quantitative estimation follow the description given above for the method of Zander and Simmer. The values for human plasma obtained with the method are corrected for extraction losses by multiplying by 1.37, since the mean recovery rate of about

<sup>3</sup> An extension of this method for the simultaneous determination of estrogens has been described by Short (1960).

5–10  $\mu$ g. progesterone added to 10–25 ml. male human plasma was shown to be 73%. In some instances it is necessary to chromatograph the progesterone eluate a second time in order to achieve sufficient purification for estimation in the spectrophotometer. In a small proportion of these cases, even the second chromatography fails to remove the impurities that absorb intensely in the 220-m $\mu$  region of the ultra-violet. According to Short and Eton (1959) the Allen correction, applied to these samples, would give a gross underestimation of the progesterone concentration. The unidentified impurities can probably be removed by acetylating the progesterone eluate with acetic anhydride and pyridine overnight. This may alter the  $R_f$  value of the impurities in a subsequent chromatography.

#### C. METHOD OF OERTEL, WEISS, AND ELK-NES (1959)<sup>4</sup>

##### *Reagents*

1. Absolute ethanol, U.S.P. grade.
2. Absolute methanol, A.R. grade, redistilled over dinitrophenylhydrazine and over Ag<sub>2</sub>O *in vacuo*.
3. *n*-Hexane, A.R. grade, purified over concentrated H<sub>2</sub>SO<sub>4</sub>, washed with bicarbonate and water, and redistilled.
4. Chloroform, A.R. grade, redistilled over K<sub>2</sub>CO<sub>3</sub>.
5. Benzene, A.R. grade, purified over H<sub>2</sub>SO<sub>4</sub>, washed with bicarbonate and water, dried, and redistilled.
6. Propylene glycol, A.R. grade.
7. Methylcyclohexane, A.R. grade, redistilled.

*Precipitation of protein.* To each plasma sample 1000 c.p.m. of radioactive progesterone is added. The plasma is thoroughly shaken for 15 minutes with 3 times its volume of absolute ethanol in a centrifuge tube or bottle. After centrifugation the extract is transferred to a second centrifuge tube or bottle and the precipitate is extracted twice with 0.5 volume of absolute ethanol each time. The combined ethanol extracts are evaporated to dryness *in vacuo* at 40°C.

*Removal of lipid material.* The residue of the ethanol extract is dissolved in 10 ml. of 70% methanol and kept at –15°C. for 15 hours before it is centrifuged for 15 minutes at 2000 r.p.m. in a refrigerated centrifuge. The supernatant is carefully removed and the precipitate washed once with 2 ml. of ice cold 70% methanol. The aqueous phase is diluted with 15 ml. of water and extracted 3 times with 15 ml. of

<sup>4</sup>The present description gives some modifications of the first publication (Oertel, 1960, personal communication).

*n*-hexane each time. The combined *n*-hexane extracts are evaporated to dryness *in vacuo*.

**Paper chromatography.** The dry residue is applied on a 4- × 12-cm. Whatman No. 1 paper strip using 3 times 0.2 ml. of methanol chloroform (1:1 v/v). In a similar fashion a 50-μg. sample of androst-4-ene-3, 17-dione is applied to the same paper strip as a reference compound, and the strip is submitted to ascending chromatography with methanol-benzene (1:9 v/v) (Oertel, 1956). Within 3 to 4 hours the free steroids are concentrated at the tip of the concentration strip. This tip, containing the extract, is cut off and inserted into corresponding cuts on propylene-glycol-methanol-impregnated paper strips (propylene glycol: methanol = 1:1.5 v/v). The paper chromatogram is developed with methyleyclohexane for 8 hours. With every paper chromatogram a 50-μg. sample of progesterone is run alongside on one strip as a reference compound. After 8 hours the paper chromatogram is removed and carefully dried.

**Elution of progesterone and purification of paper eluates.** The spots of progesterone and androst-4-ene-3,17-dione on the standard strip are located by the UV scanning technique and the  $R_T$  value of progesterone is determined. The area of the sample strip exhibiting the same  $R_T$  value as that of the standard progesterone is eluted with methanol. Likewise, two pieces of paper from a blank strip corresponding in size and location to that of the sample strip are eluted, and 10 μg. of progesterone are added to one of the blank eluates. The three eluates are then evaporated to dryness *in vacuo*. The dry residues are redissolved in 10 ml. of benzene and extracted once with 5 ml. of 0.5% sulfuric acid and twice with 5 ml. of water each time. The benzene solutions are evaporated to dryness *in vacuo*.

**Quantitative estimation.** The residue of the plasma sample is dissolved in 1 ml. of methanol, and 0.1 ml. of this solution is plated and counted in a windowless counter. The remaining methanol solution is evaporated to dryness. The dry residue of the three extracts—sample, standard, and paper blank—are dissolved in 0.5 ml. of reagent, freshly prepared from 1 volume of 80% ethanol and 2 volumes of concentrated  $H_2SO_4$ . The reaction mixture is warmed for 15 minutes at 60°C., cooled, and transferred into microcuvettes. The absorption is measured at 260, 290, and 320  $m\mu$ , using the paper blank as a reference. The maximum absorbaney at 290  $m\mu$  is corrected by a formula similar to that of Allen, as modified by Brown:

$$O.D._{290 \text{ corr.}} = 2 \times O.D._{290} - O.D._{260} - O.D._{320}$$

The amount of progesterone present in the sample is then calculated

from the corrected absorbancy of the standard. Losses are corrected by establishing the recovery of the added radioactive progesterone. Additional information may be obtained by determination of the UV absorption of the purified paper eluates prior to the color reaction, using the absorbancy at 225, 240, and 255  $m\mu$ .

### VIII. Application of Methods

The practical usefulness of any method depends, evidently, upon its sensitivity and upon the concentration of the substance to be determined in the test material. As stated in Section VI, D, the most sensitive methods presently available permit the determination of 0.5 to 1.0  $\mu g$ . of progesterone. The following discussion on the progesterone concentrations encountered in human tissues and plasma under a variety of physiological conditions may be useful in selecting adequate quantities of tissue and plasma for a reliable determination.

#### A. TISSUE

The range of progesterone concentration in various human tissues and the amounts of tissue needed for a reliable estimation are listed in Table VII. Normal values for the total amount of progesterone in

TABLE VII

RANGE OF PROGESTERONE LEVELS IN DIFFERENT HUMAN TISSUES

Tissue	Approximate range of progesterone levels <sup>a</sup> ( $\mu g.$ /per gram wet tissue)	Grams of tissue needed for reliable analysis with the most sensitive methods presently available
Ripe graafian follicle	1.0-150.0	1.0
Corpus luteum	2.0- 50.0	0.5-1.0
Placenta	1.0- 6.0	5.0-10.0
Fat tissue	<0.1- 2.0	10.0-20.0
Uterine mucosa	<0.1	Unknown
Uterine muscle	<0.1	Unknown

<sup>a</sup> Single levels in the given range are dependent on the age of the tissue and the physiological state of the woman. Data reported by Zander *et al.* (1958).

graafian follicles before ovulation and in corpora lutea during the cycle as well as in placenta during pregnancy are shown in Figs. 5 and 6.



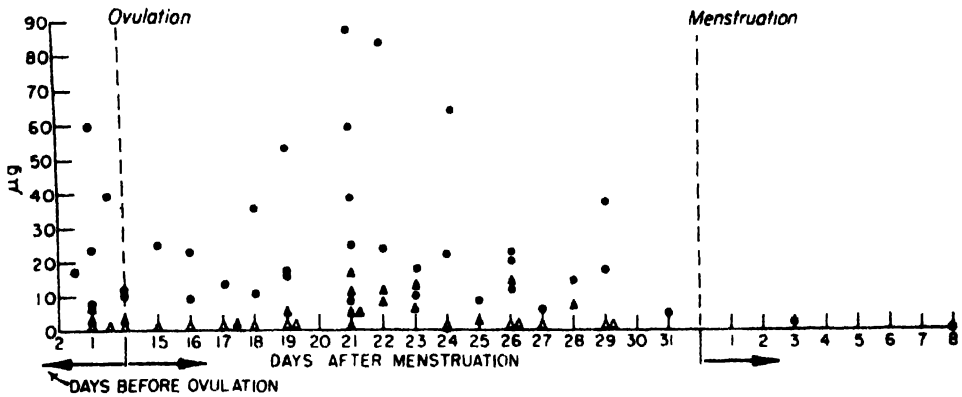


FIG. 5. Total amounts of progesterone (dots) and of combined 20 $\alpha$ -hydroxypregn-4-en-3-one fraction (solid triangles) in each of 7 follicles obtained 1 to 2 days before ovulation and 32 corpora lutea of the cycle. The open triangles represent values which were too low for an exact quantitative determination. From Zander *et al.* (1958).

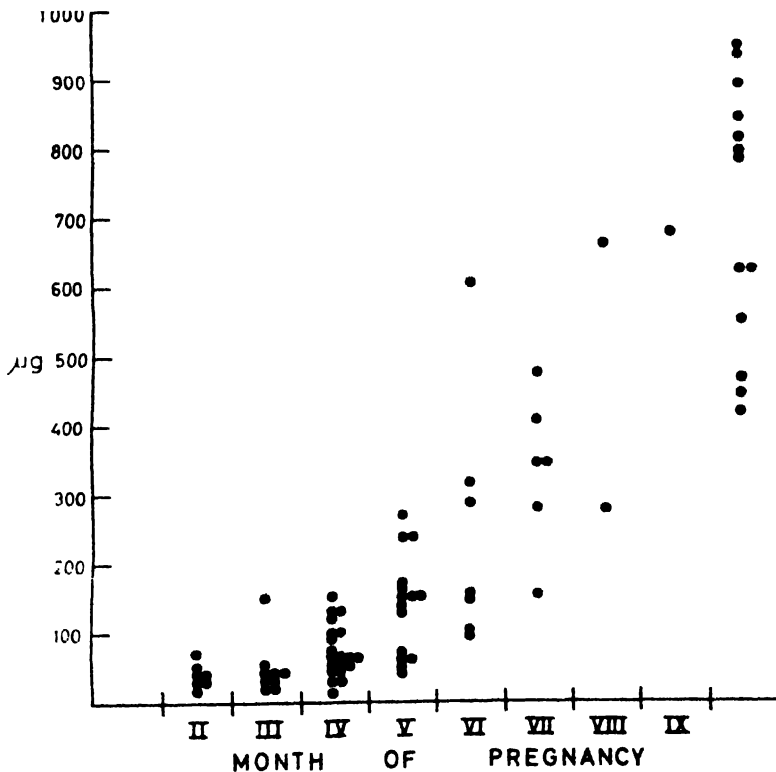


FIG. 6. Total amounts of progesterone in each of 80 human placentae in the second to the tenth month of pregnancy. From Zander and von Mjinstermann (1956).

Because of the relatively high concentration in progesterone-secreting organs, reasonable results can be expected with comparatively small tissue quantities. Thus it suffices to submit to analysis a single corpus luteum or even parts thereof. A relatively small sample of fat tissue will also yield satisfactory results. Other tissues, e.g., target organs, contain normally such minimal amounts of progesterone that the whole available tissue is not sufficient for quantitative estimation of the hormone.

### B. PLASMA

Whereas the progesterone determinations in tissues are, under most circumstances, of strictly scientific interest, those in plasma may assume considerable practical significance. Deviations of the normal progesterone level in blood may give helpful diagnostic indications, e.g., in cases of corpus luteum insufficiency threatening abortion or in the presence of endocrinologically active neoplasms. One can visualize also that the observation of the progesterone plasma concentration during stimulation with gonadotropic hormone may lead to valuable diagnostic information. The first requirement for this kind of study is an exact knowledge of the progesterone concentration in the blood under physiological conditions. Secondly, it would be necessary to have information on the physiological fluctuations, e.g., the diurnal variations; thirdly, a method must be so sensitive that repeated progesterone determinations can be made with a reasonably small volume of blood.

The range of progesterone levels during the menstrual cycle and pregnancy of healthy women are listed in Table VIII together with the blood quantities necessary for reliable determinations with present

TABLE VIII  
RANGE OF PROGESTERONE LEVELS IN HUMAN PERIPHERAL PLASMA

Physiological state	Approximate range of progesterone levels ( $\mu\text{g.}/100 \text{ ml. plasma}$ )	Milliliters of blood needed for reliable analysis with the most sensitive methods presently available
Cycle	<1.0– 5.0 <sup>a</sup>	100–>100
Pregnancy (first half)	<5.0–10.0 <sup>b</sup>	50–100
Pregnancy (second half)	5.0–40.0 <sup>b</sup>	25–50

<sup>a</sup> Data reported by Zander (1955) and Oertel *et al.* (1959).

<sup>b</sup> Data reported by Zander (1954, 1955) and Short (1958, 1960).

methods. Figure 7 shows normal values in the peripheral blood plasma during pregnancy, beginning with the eleventh week. It is evident that in the luteal phase of the cycle and in the first half of pregnancy, the amount of peripheral (arm vein) blood needed for reliable analysis is

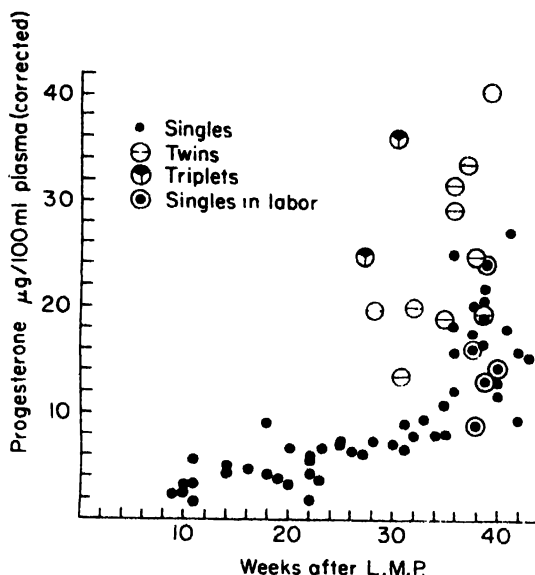


FIG. 7. Concentration of progesterone in plasma of pregnant women. Results are corrected for extraction losses and expressed as  $\mu\text{g}$ . progesterone per 100 ml. of blood. From Short (1961).

still too high to make repeated determinations feasible without endangering the well being of the patient. For both scientific and diagnostic problems, repeated determinations are most desirable and thus every effort must be made to develop methods with greater sensitivity.

### IX. Suggestions for Further Development of Methods

According to the conclusion reached in Section VIII, B, the chief desideratum for further progress in this field remains the development of methods with increased sensitivity without loss of reliability. Especially for *clinical investigations*, a method would be required which needs not more than 5–10 ml. of blood for a single determination. For such a goal the sensitivity of the present methods would have to be increased ten- to twentyfold. It is rather doubtful that the sensitivity of any of the quantitative procedures described in Section IV can be increased to that extent. An exception is the new fluorescence reaction of Touchstone

and Murawec (1960) which may be helpful for the development of more sensitive routine methods, provided it is possible to maintain a sufficient reliability using this method.

It would be promising to find ways and means for the formation of radioactive derivatives of isolated progesterone with radioactive ketonic reagents, e.g., with  $C^{14}$ -dinitrophenylhydrazine or  $C^{14}$ -thiosemicarbazide. Such derivatives could be easily characterized by their chromatographic mobility, and the quantitative estimation of progesterone could be performed by measuring the radioactivity of the derivative.

By utilizing the same principle it should be possible to prepare a radioactive derivative, e.g., by acetylation with  $C^{14}$ -acetic anhydride, after chemical or enzymatic reduction of the keto group at C-20.

It seems possible that this or a similar approach would increase the sensitivity of the methods considerably without loss of reliability.

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## Chapter 4

# Pregnanediol and Pregnanetriol

ARNOLD I. KLOPPER

I. The Metabolic Precursors of Pregnanediol and of Pregnanetriol . . . .	140
A. The Structural Relationship of Progesterone and Pregnanediol . . .	140
B. The Structural Relationship of 17 $\alpha$ -Hydroxyprogesterone and Pregnanetriol . . . . .	141
C. Precursors Other Than Progesterone and 17 $\alpha$ -Hydroxyprogesterone . .	142
II. Organs Producing Pregnanediol and Pregnanetriol Precursors . . . . .	142
A. Ovary . . . . .	142
B. Placenta . . . . .	143
C. Adrenal . . . . .	143
III. The Occurrence of Pregnanediol and Pregnanetriol . . . . .	144
A. Blood . . . . .	144
B. Bile . . . . .	144
C. Feces . . . . .	144
D. Fat . . . . .	145
E. Urine . . . . .	145
IV. Quantitative Relationships between Hormone Production and Metabolite Excretion . . . . .	146
A. Progesterone and Urinary Pregnanediol . . . . .	146
B. 17 $\alpha$ -Hydroxyprogesterone and Pregnanetriol . . . . .	146
V. The Development of Methods for the Assay of Pregnanediol and Pregnanetriol in Urine . . . . .	147
A. Pregnanediol . . . . .	147
B. Pregnanetriol . . . . .	150
VI. A Critical Evaluation of the Principles of Published Methods of Assay .	151
A. Criteria by Which Chemical Assays of Urinary Steroids Should Be Judged . . . . .	151
B. The Application of Reliability Criteria to Assay Methods . . . . .	153
VII. Recommended Methods . . . . .	156
A. Methods for the Assay of Urinary Pregnanediol . . . . .	156
B. Methods for the Assay of Urinary Pregnanetriol . . . . .	159
VIII. The Clinical Applications of the Assay of Pregnanediol and Pregnanetriol	161
A. Ovarian Studies . . . . .	161
B. Placental Studies . . . . .	164
C. Adrenal Studies . . . . .	164
IX. Future Developments in the Assay of Pregnanediol and Pregnanetriol .	165
References . . . . .	167

## I. The Metabolic Precursors of Pregnanediol and of Pregnanetriol

In Fig. 1 are shown the structural formulas of the inert urinary metabolites to be discussed in this chapter together with the formulas

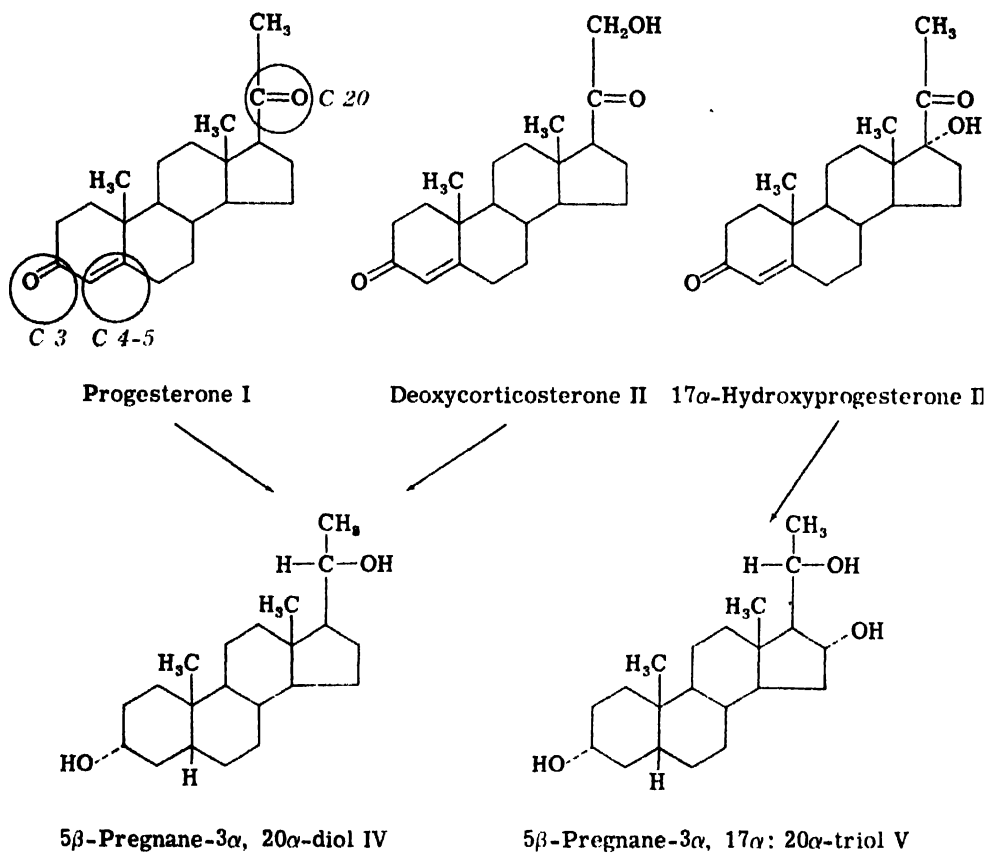


FIG. 1. The metabolic precursors of pregnanediol and pregnanetriol.

of the active hormones from which they are presumed to be derived. These urinary end products are 5 $\beta$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol (IV) and 5 $\beta$ -pregnane-3 $\alpha$ , 17 $\alpha$ , 20 $\alpha$ -triol (V).

### A. THE STRUCTURAL RELATIONSHIP OF PROGESTERONE AND PREGNANEDIOL

The characteristic features of progesterone (I) are ringed in Fig. 1. They are the keto group at C-3, the unsaturated bond between C-4 and



C-5, and the keto group at C-20. All three of these groups are subject to metabolic reduction. It is by no means certain in which order these reductions take place but it is convenient to consider first the effect of saturating the double bond between C-4 and C-5. This gives rise to the compound pregnanediolone. As a result of the saturation of the double bond C-5 now has a hydrogen atom attached to it. There are in fact two pregnanediolones depending upon the orientation in space of this hydrogen atom. If it is on the same side of the nucleus as the methyl groups at C-18 and C-19 (*cis*), the compound is designated  $5\alpha$ -pregnanediolone, the parent compound of the allopregnane series. If the hydrogen group is opposite to (*trans*) the angular methyl groups the compound is designated  $5\beta$ -pregnanediolone. Pregnanediol itself is a derivative of the  $5\beta$  series. The keto group at C-3 is probably reduced to a hydroxyl group at the same time as the saturation of the double bond occurs. This hydroxyl group may again be either *cis* or *trans* in orientation. There are thus four pregnanolone isomers, one of which,  $5\beta$ -pregnan-3 $\alpha$ -ol-20-one, occurs in appreciable quantities in urine. Finally the C-20 keto group is also reduced to either an  $\alpha$ - or a  $\beta$ -hydroxyl group. It follows that at least eight pregnanediol isomers may occur. Fortunately there is clear evidence that in man the metabolic pathway is specifically directed toward the production of  $5\beta$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol, the compound to which the common name pregnanediol is applied. Another pregnanediol isomer  $5\alpha$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol, has been isolated in small quantities from pregnancy urine by Hartmann and Locher (1934), and it is likely that small amounts of other isomers are normally present in urine. No methods exist for their separate assay. They are not known to have any particular physiological significance and are not present in sufficient amount noticeably to affect the assay of  $5\beta$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol although they may be present to some degree in the final residues which are measured.

#### B. THE STRUCTURAL RELATIONSHIP OF 17 $\alpha$ -HYDROXYPROGESTERONE AND PREGNANETRIOL

As can be seen in Fig. 1, 17 $\alpha$ -hydroxyprogesterone (III) differs from progesterone only in having the hydrogen atom on C-17 replaced by an  $\alpha$ -orientated hydroxyl group. There appears to be no clear evidence to suggest how, if at all, this influences the metabolic reduction of the  $\alpha,\beta$ -unsaturated ketone in ring A or the C-20 carbonyl group. The indications are that metabolism follows the same lines as that of progesterone, a similar series of intermediates and isomers being formed which differs from the progesterone series only in the possession of the extra

hydroxyl group at C-17. Thus pregnan-17 $\alpha$ -ol-3, 20-dione is an accepted intermediate and pregnan-3 $\alpha$ , 17 $\alpha$ -diol-20-one has been isolated from urine.

### C. PRECURSORS OTHER THAN PROGESTERONE AND 17 $\alpha$ -HYDROXYPROGESTERONE

Horwitt *et al.* (1944) have produced evidence that the administration of deoxycorticosterone (II) gives rise to urinary pregnanediol. This does not of course prove that some deoxycorticosterone is not first converted to progesterone. Indeed it is assumed by Dorfman (1954) in his analysis of deoxycorticosterone metabolism that the pregnanediol excreted from this compound is formed via progesterone. Deoxycorticosterone has been isolated from adrenal tissue (Hechter and Pincus, 1954), but it does not appear to be an adrenal hormone in its own right and there is no reason to suppose that it contributes any substantial fraction to the urinary pregnanediol. As might be expected, 17-hydroxy-11-deoxycorticosterone is a precursor of urinary pregnanetriol but here again the same observations hold good as for deoxycorticosterone and pregnanediol. In practice it has appeared safe to ignore all precursors of pregnanediol and pregnanetriol other than progesterone and 17 $\alpha$ -hydroxyprogesterone.

## II. Organs Producing Pregnanediol and Pregnanetriol Precursors

### A. OVARY

Progesterone is produced by the corpus luteum in the ovary and has been isolated from luteal tissue in the sow (Butenandt *et al.*, 1934). It is presumed to originate from the granulosa lutein cells of the corpus luteum but it is possible that the cells of the theca interna are also capable of producing progesterone.

There is clear evidence for the existence of androgenic activity in the ovaries of birds and material having androgenic activity has been extracted from mammalian ovaries but no pure substance has been isolated.

Some ovarian tumors, such as arrhenoblastomata, are known to cause masculinization but it is sometimes difficult to exclude aberrant adrenal tissue as the cause of virilization. Deanesly (1938) associated the androgenic activity of the ovary with the theca interna. Recent work has suggested the possibility of an anabolic pathway for estrogens leading

from progesterone through  $17\alpha$ -hydroxyprogesterone and  $\Delta^4$ -androstene-3, 17-dione (Baggett *et al.*, 1959). Both the latter have in fact been isolated from mare follicular fluid (Short, 1959). It is possible therefore that  $17\alpha$ -hydroxyprogesterone may be produced in the ovary as an intermediate in the anabolism of its characteristic hormones and that, on occasion, urinary pregnanetriol arises as the metabolite of an ovarian precursor. At present these possibilities are speculative but more might be learned from pregnanetriol assay in patients suffering from virilism. Even where the masculinization is of ovarian origin as in the Stein-Leventhal syndrome the presumed androgen may well give rise to metabolites such as pregnanetriol.

### B. PLACENTA

Progesterone has been isolated from human placentae (Pearlman and Cereco, 1952). Tissue culture experiments suggest that it is produced by the syncytiotrophoblast.

### C. ADRENAL

The elaboration of the characteristic corticoids of the adrenal appears to proceed in the zona fasciculata of the adrenal cortex. Figure 2 shows the scheme of corticosteroidogenesis suggested by Hechter (1955), a view which has gained widespread acceptance. This emphasizes the central role played by progesterone and  $17\alpha$ -hydroxyprogesterone in the

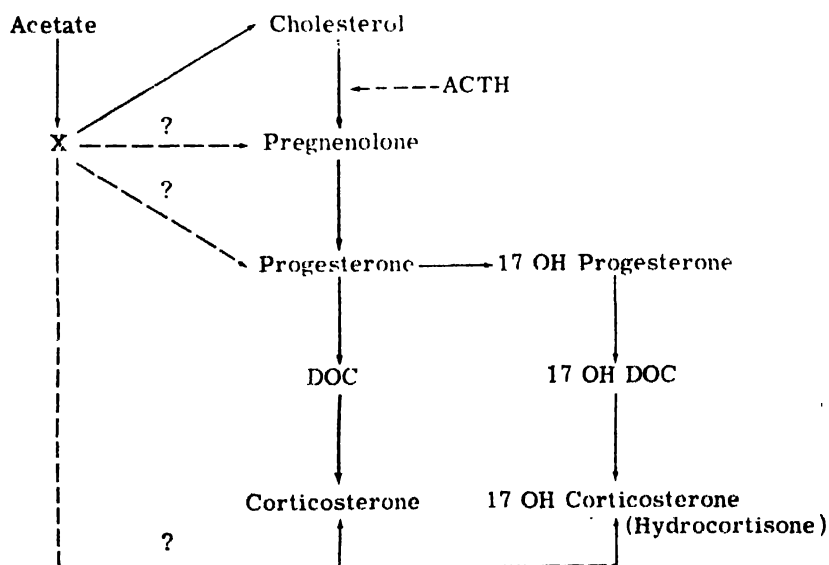


FIG. 2. Scheme of corticosteroidogenesis.

elaboration of adrenal hormones. A significant fraction of the pregnanediol normally present in the urine of both sexes has been shown to come from the adrenal, presumably as an incidental catabolite of progesterone acting, not as an active hormone, but as a central intermediate in corticosteroidogenesis. An association between the levels of urinary pregnanediol and the physiological activity of the adrenal has been demonstrated by Klover and his associates (1957).

It has been shown by Bongiovanni and his co-workers (1954) that pregnanetriol is excreted in large quantities in the urine of patients with virilizing adrenal hyperplasia. It has been suggested that in these patients a defect exists in the anabolism of  $17\alpha$ -hydroxyprogesterone to corticoids. There is an increased secretion of adrenocorticotropin with resulting overproduction of virilizing steroids. Bongiovanni *et al.* (1954) were able to show that the administration of cortisone leads to a fall in the excessive output of pregnanetriol by these patients.

### III. The Occurrence of Pregnanediol and Pregnanetriol

#### A. BLOOD

A method for the estimation of pregnanediol in blood has been published (Sommerville and Deshpande, 1958). It appears to be a preliminary study soon to be superseded, but these workers have indicated that pregnanediol is present in the plasma and that at least some of it is in the same form as that in which it occurs in urine, i.e., as a glucuronoside. No experiments appear to have been done to determine whether any pregnanediol occurs in simple solution as the free alcohol, or in some sort of association with plasma proteins or whether the red cells have any appreciable pregnanediol content.

#### B. BILE

Rodgers and McLellan (1951) found pregnanediol in the bile of patients given large doses of progesterone by mouth. In a series of measurements of biliary pregnanediol following the injection of progesterone, Klover and Macnaughton (1959) showed that 3-5% of the progesterone was present as pregnanediol in the bile. All the biliary pregnanediol they found was present in the conjugated form.

#### C. FECES

Pregnanediol has been isolated from the feces of a pregnant woman by Klover and Macnaughton (1959). It was present as the free steroid.

They surmised that an enterohepatic circulation of pregnanediol might take place. Some of the water-soluble conjugated pregnanediol formed in the liver is secreted into the bile, and in the gut some of this is hydrolyzed, presumably by bacteria, to the free insoluble alcohol and excreted in the feces. Any unhydrolyzed soluble material is reabsorbed and excreted in the urine.

#### D. FAT

The work of Davis and Plotz (1958) has shown that progesterone or its metabolites may be stored for a considerable length of time in the fat compartments of the body. It is difficult to assess what proportion may be thus stored but it is unlikely to exceed 30%, which is the fraction of injected radioactive progesterone they were unable to account for in other biological fluids.

#### E. URINE

Figure 3 shows the structural formula of pregnanediol glucuronoside, the water-soluble compound which occurs in urine. Some steroids, such

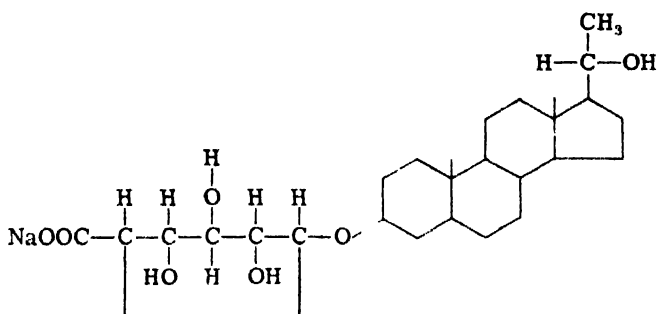


FIG. 3. The structural formula of pregnanediol glucuronoside.

as estriol, also occur in urine as sulfates but pregnanediol sulfate has never been isolated. Pregnanediol glucuronoside may readily be hydrolyzed in urine, probably by bacterial action, releasing the free, insoluble steroid. Partial hydrolysis of the conjugate was one of the main sources of error in earlier methods for the estimation of urinary pregnanediol which depended on the isolation of the conjugated material. As the free alcohol is produced by incubating urine with  $\beta$ -glucuronidase, it is clear that some, if not all, the pregnanetriol in urine is also present as a glucuronoside.

#### IV. Quantitative Relationships between Hormone Production and Metabolite Excretion

Almost invariably estimates of urinary metabolites are made in order to get an impression of the rate of production of the original hormone. For this purpose it is useful to know the ratio between active hormone and inert metabolite, and it is essential that some fairly constant relationship should exist between the two. It must be admitted that the constancy of the relationship between progesterone production and urinary pregnanediol is open to question and that there is very little evidence bearing on the ratio between  $17\alpha$ -hydroxyprogesterone and urinary pregnanetriol.

##### A. PROGESTERONE AND URINARY PREGNANEDIOL

A good deal of work has been done on the recovery of urinary pregnanediol after the injection of progesterone. In a series of 16 experiments Kloppe and Michie (1956) found that, when progesterone was injected, the recovery of material as urinary pregnanediol ranged from 5 to 17% with a mean recovery of 13%. In the majority of cases the conversion fell into a very narrow range between 11 and 14%. It was at one time suggested that the conversion of progesterone to urinary pregnanediol was enhanced in pregnancy or when a secretory endometrium was present but the experiments of Kloppe and Michie (1956) and of Davis and Plotz (1958) have disposed of this idea. The liver appears to be the main site for the metabolic reduction of progesterone to pregnanediol. This reaction has indeed been demonstrated *in vitro* with liver preparations by Taylor (1955). Experimental findings suggest that even in severe liver disease the capacity of the liver to effect the reduction of progesterone to pregnanediol is not affected.

##### B. $17\alpha$ -HYDROXYPROGESTERONE AND PREGNANETRIOL

Very little is known about the quantitative relationship between the  $17\alpha$ -hydroxyprogesterone produced in the body and the pregnanetriol excreted in the urine. Bongiovanni *et al.* (1954) showed that an increase in urinary pregnanetriol excretion occurred when  $17\alpha$ -hydroxyprogesterone was injected into patients with adrenal hyperplasia but not in normal people. Pregnanetriol is present in the urine of normal subjects so that it is likely that a proportion of  $17\alpha$ -hydroxyprogesterone is thus catabolized. Evidently, however, the main metabolic pathway for  $17\alpha$ -

hydroxyprogesterone is in corticosteroidogenesis, and the normal urinary output of pregnanetriol is of no particular physiological significance. Bartter (1951) has postulated that the adrenogenital syndrome is due to a defect in glucogenic corticoid synthesis. It is in patients with this condition that a large excretion of pregnanetriol may indicate blockage in the normal pathway of  $17\alpha$ -hydroxyprogesterone.

## V. The Development of Methods for the Assay of Pregnanediol and Pregnanetriol in Urine

### A. PREGNANEDIOL

#### 1. Isolation and Identification

In 1929 Marrian isolated a crystalline solid from the unsaponifiable residue of ether extracts of late pregnancy urine. He showed this compound to be a dihydroxy alcohol. Butenandt in 1930 and 1931 worked out its empirical formula and proved that it was a saturated dihydroxy compound with 4 hydrogenated rings in the molecule. He pointed out its relationship to the sterols and bile acids and named it pregnanediol. In 1934 Butenandt and Schmidt demonstrated a connection between pregnanediol and the active hormone progesterone by synthesizing the latter from pregnanediol.

In 1936 Odell and Marrian extracted a combined form of pregnanediol from pregnancy urine. They noted that the free steroid could be obtained by hydrolysis of this compound with hot acid. Later in the same year Venning and Browne (1936) isolated the water-soluble combined form of pregnanediol from butanol extracts of late pregnancy urine. They were able to show that it was present as the monosodium salt of pregnanediol glucuronic acid. In 1937 Venning and her colleagues demonstrated a physiological connection between progesterone and pregnanediol by isolating the latter from the urine of subjects injected with progesterone and of normal women in the luteal phase of their menstrual cycle. It therefore appeared that the quantity of pregnanediol in the urine might serve as an index of the production of progesterone in the body. Attention was accordingly directed to the elaboration of methods for assaying urinary pregnanediol.

#### 2. The Growth of Quantitative Methods

*a. Gravimetric Methods.* The first attempt at a quantitative determination of urinary pregnanediol was made in 1937 when Venning published a method based on her technique for isolating the sodium pregnanediol glucuronoside complex. The complex was extracted from urine with

butanol which was washed with strong alkali solution. The residue left after evaporation of the butanol was then twice precipitated from aqueous solution by the addition of acetone. Finally the precipitate was dried and weighed. She concluded that her method recovered a proportion of sodium pregnanediol glucuronoside which grew larger as the amount of steroid originally present in the urine grew larger, and she devised a formula to calculate the true content. The minimum which could be measured with any degree of accuracy was 5 mg. of pregnanediol per 500 ml. of urine.

The publication of this method stimulated research. Many quantitative analyses were performed on urine from both pregnant and non-pregnant women. As commonly occurs with new methods, workers doing such assays introduced minor modifications of their own. Several years, however, elapsed before the limitations of this method were sufficiently clear to stimulate the search for more refined methods of assay. Instead the basic work on the physiological role of progesterone was pursued using this new tool. Indeed, today much of our information on the excretion of pregnanediol in health and disease is still based on results obtained by this method.

By 1938 Venning had done more than 2000 pregnanediol determinations and noted some of the shortcomings of the technique. She found that the final residue was often grossly impure and that spontaneous hydrolysis of the glucuronoside complex tended to take place before the urine could be processed. The limitations of the Venning procedure led to many attempts to improve upon it. Maughan *et al.* (1938) hydrolyzed the glucuronoside complex isolated by the Venning technique and measured the glucuronic acid set free by a modification of the Tollens naphtharesorcinol reaction. Allen and Viergiver in 1941 and Hechter in 1942 carried this idea further, improving on the Venning precipitation procedure and producing more refined ways of measuring the liberated glucuronic acid.

*b. Acid Hydrolysis and Precipitation.* A major advance in the technique of pregnanediol assay was introduced by Astwood and Jones in 1941. They did not extract the conjugated steroid from urine but first hydrolyzed it by boiling the urine with hydrochloric acid. The free steroid was then extracted with toluene and the acidic impurities removed by treatment with sodium hydroxide. The residue was further purified by repeated precipitation from ethanol using dilute sodium hydroxide or water. The final precipitate was dried and weighed.

*c. The Use of the Sulfuric Acid Color Reaction.* A further improvement in assay technique occurred in 1941 when Talbot *et al.* found that pregnanediol gave rise to a yellow color with sulfuric acid. They applied



this to the material isolated by the Astwood and Jones process, comparing the density of the colors thus produced with that from known weights of pure pregnanediol.

*d. Further Refinements and Simplifications.* Sommerville *et al.* (1948a), studied critically all the steps which had been evolved—hydrolysis, extraction, alkali treatment, precipitation, and sulfuric acid color reaction—and suggested improvements in each. By now, however, the assay method had grown too cumbersome for routine clinical purposes and various short cuts were evolved, notably by Sommerville *et al.* (1948b) and Guterman and Schroeder (1948). These reduced the precautions taken to ensure the quantitative recovery of steroid and so detracted from the reliability of the results. Some investigations of obstetric pathology and metabolic experiments produced conflicting results. It became evident that it would be necessary to design methods of pregnanediol determination which would be more reliable and less laborious if useful clinical information was to be obtained, especially in nonpregnant states.

*e. Enzyme Hydrolysis.* In order to avoid the contamination of extracts by the pigments produced in the acid hydrolysis of urine, Cohen (1951) published a method in which hydrolysis was effected by  $\beta$ -glucuronidase extracted from calf spleen. With subsequent methods, however, it has been possible to eliminate the unwanted chromogens resulting from acid hydrolysis. As enzyme hydrolysis is more laborious and because aberrant enzyme inhibitors may occur in urine, modern methods have favored acid hydrolysis wherever possible.

*f. Chromatographic Techniques.* The introduction of chromatography to the field of steroid chemistry led to the application of this technique to pregnanediol assay. In 1947 Huber published a method in which the neutral toluene extract of hydrolyzed urine was absorbed on alumina, the pregnanediol being eluted with ether. This method was modified and greatly improved by de Watteville *et al.* a year later (1948). Other chromatographic techniques were published in America during 1952. Stimmel *et al.* (1952) used an alumina adsorption technique followed by precipitation from ethanol. Later in the same year a more definitive technique was published by Chaney and his collaborators (1952). They used a chromatographic column consisting of Celite and calcium carbonate from which the pregnanediol was eluted with a mixture of methanol and toluene.

The final residue obtained by all these methods is impure. Some of the contaminants are intensely chromogenic with sulfuric acid and even small amounts of impurity may give rise to massive overestimates. These considerations led Kloppe and his associates (1955) to introduce

an alumina adsorption chromatography method in which the potentialities of this technique were more fully exploited. This was done by designing a solvent system such that fast-running compounds such as 17-oxosteroids were first removed from the column and that trihydroxy or tetrahydroxy compounds were left on the column after the removal of pregnanediol. Pregnanediol was further separated from contaminants by acetylation and repeat chromatography in a different system. At the same time Kloppe *et al.* introduced a further purification step by oxidation of the neutral toluene extract of hydrolyzed urine, and later also studied the sulfuric acid color reaction in some detail (Kloppe, 1956). A different line was followed by Bongiovanni and Eberlein (1958) who use paper chromatography to produce pregnanediol free from aberrant sulfuric acid chromogens.

The methods mentioned in this chapter are a selection from the large number of "new" techniques produced each year. Most of these are minor modifications of existing practices, e.g., the techniques of Westphal (1944), Furujhelm (1953), Trolle (1955), Verboom (1957), and Jensen (1958). They have not been applied outside the laboratories of their originators.

## B. PREGNANETRIOL

### 1. Isolation and Identification

Butler and Marrian in 1937 were the first workers to isolate pregnanetriol from urine. They found the steroid in the urine of two women suffering from the adrenogenital syndrome. They were able to do a structural identification of the molecule and to list its properties. This publication appears to have roused very little interest and it was not until 1953 that Cox and Marrian showed that pregnanetriol was also present in urine from normal men and assessed the normal excretion to be 1-2 mg./24 hours.

### 2. The Growth of Methodology

As its precursor in the body was not a hormone with a clearly defined physiological role, there was no stimulus for the development of methods for the quantitative assay of urinary pregnanetriol. Also pregnanetriol is not stable to the usual hot acid hydrolysis employed in steroid analysis and its quantitative estimation was delayed until enzyme preparations were more readily available. Two events have in recent years given fresh impetus to methodological research in this field. The first was the realization of the central role of 17 $\alpha$ -hydroxyprogesterone in corticosteroidogenesis in the adrenal and its possible role

in ovarian physiology (Section II, A and C). The second was the demonstration by Bongiovanni *et al.* in 1954 that patients with virilizing adrenal hyperplasia excrete large amounts of pregnanetriol in the urine. Bongiovanni and Clayton published a quantitative method in 1954. The urine was incubated with  $\beta$ -glucuronidase and the pregnanetriol extracted with benzene. The extract was then fractionated on alumina, separating off pregnanediol and other steroids. The pregnanetriol was finally estimated by the sulfuric acid color reaction. Two later methods (Stern, 1957; Bongiovanni and Eberlein 1958) both used the same principles, alumina chromatography and sulfuric acid color reaction. De Courcy (1956) isolated pregnanetriol by paper chromatography and detected the steroid as a blue spot with a special trichloroacetic acid reagent. Her technique is primarily qualitative but a rough quantitative estimate can be obtained by examining the fluorescence of the spot. Herrmann and Silverman (1957) used chromatography on Florisil and estimated by the Zimmermann reaction the 17-oxosteroid liberated as a result of oxidation of the pregnanetriol fraction with sodium bismuthate. Cox (1959) measured the acetaldehyde produced when pregnanetriol was oxidized with periodic acid, having first been isolated by paper chromatography. Fotherby (1960) has produced a somewhat more refined version of Stern's method, making better use of the capacity of alumina columns to resolve complex steroid mixtures.

## VI. A Critical Evaluation of the Principles of Published Methods of Assay

### A. CRITERIA BY WHICH CHEMICAL ASSAYS OF URINARY STEROIDS SHOULD BE JUDGED

Certain data should always be published with any new method in order to enable the reader to evaluate it. Unfortunately few publications do in fact give such data fully. It is essential to give adequate findings on specificity, accuracy, precision, and sensitivity. These criteria have been fully discussed by Borth (1952) and by Loraine (1957) but they are sufficiently important to warrant reiteration.

#### 1. *Specificity*

Specificity is the degree to which only the material to be assayed is measured in the final determination. There is no known specific reaction for pregnanediol or pregnanetriol. The specificity of any assay method therefore depends to a large extent on how completely the steroid can be separated from the other constituents of urine and from the substances

employed in their isolation. In practice such isolation has always to be balanced against the losses incurred with each purification step. The bad compromise is often excused by the statement that "it is good enough for clinical purposes." Clinical purposes are in fact no less exacting than any other scientific purpose.

## 2. Accuracy

Accuracy is the nearness with which a given analytical result approaches the "true" result. The accuracy of a method for urinary pregnanediol or pregnanetriol can be assessed by the percentage of the steroid recovered when measured amounts are added to the urine. As both these steroids are excreted in the conjugated form, true recovery experiments should consist of the addition of accurately measured amounts of the conjugates. These are, however, very difficult to obtain in a satisfactorily pure state. Most of the recovery experiments have in fact been done by the addition of measured amounts of the free alcohols to urine. This is legitimate providing it is clearly realized that such experiments presuppose that the conjugate is completely hydrolyzed in the method used and that hydrolysis does not involve any destruction of the steroid. In a critical review of methods for measuring steroids Marrian (1956) suggested that recoveries above 75% could be regarded as satisfactory.

## 3. Precision

Precision can be defined as the degree by which duplicates differ from one another. It is usually expressed as the standard deviation of replicate determinations. Marrian (1956) suggested that a standard deviation of 10% at optimal steroid concentrations was a reasonable precision at which to aim.

## 4. Sensitivity

Sensitivity is the smallest weight of material which can accurately be recovered. It has sometimes been assumed that the sensitivity of a method is the smallest quantity of material it can detect. This may give a falsely favorable impression of sensitivity. Some color reactions, e.g., the Kober reaction, can detect very small amounts of material. In practice such small amounts cannot consistently and fully be distinguished either from nonspecific background color or from very slightly different amounts of the authentic substance; amounts which may differ very little in absolute terms, yet form a very large percentage of the total being measured. The sensitivity of a urinary method is related to the volume of urine which is used. Sommerville *et al.* (1948a) could satisfactorily recover 0.4 mg. of pregnanediol when added to 500 ml. of urine. Such a

quantity is, however, too small to measure by their method when added to a full normal 24-hour specimen of urine, the true sensitivity being approximately 2.0 mg./24-hour urine sample. All sensitivity statements should, therefore, be related to the basic physiological unit of a 24-hour urinary output.

### 5. Convenience

Convenience is measured by the time taken to carry out a method, the skill required, and the materials consumed. The convenience of a method is also largely to be judged in terms of the purpose for which assays are undertaken. The somewhat laborious procedure of Sommerville *et al.* (1948a) is suitable for occasional acute experiments but not for the larger series of determinations necessary to give a reliable account of the physiological variations of pregnanediol excretion in the normal menstrual cycle.

## B. THE APPLICATION OF RELIABILITY CRITERIA TO ASSAY METHODS

### 1. *Methods Based on the Isolation of the Conjugated Steroid from Urine*

These methods are for the present largely out of use and do not therefore warrant detailed consideration. They are liable to irregular losses of conjugate by spontaneous hydrolysis as shown by Bucher and Geschickter (1940). The water-soluble conjugates are more difficult to purify than the free steroids. Large volumes of urine have to be used. The very polar solvents which have to be used extract a large bulk of unwanted material from the urine. It is clear that these impurities are often incompletely removed in the Venning process by reason of the need for a melting-point control of the end product and of the considerable range (11°C.) over which the end point is allowed to vary. The introduction of a correction formula for an estimated loss during the assay merely multiplies the assumptions on which the final figure is based. The precipitation of sodium pregnanediol glucuronoside is incomplete as has been shown by the work of Westphal (1944). It is likely that most of the impurities present in the final residue are glucuronosides in which event the measurement of glucuronic acid liberated on hydrolysis reduces the specificity of the estimation. Finally the poor sensitivity of methods involving the measurement of pregnanediol glucuronoside is a major disadvantage from the point of view of investigations on nonpregnant subjects.

### 2. *The Hydrolysis of Steroid Conjugates*

Acid hydrolysis sets free in the urine a great variety of pigments and other material chromogenic with sulfuric acid. These substances are

readily extracted from the aqueous phase by organic solvents; they may be exceedingly difficult to remove completely. Some are intensely chromogenic and even small traces may give rise to gross overestimation. Enzyme hydrolysis is much more specific and does not appear to give rise to the formation of artifacts or to the destruction of steroids. Enzyme hydrolysis is essential for the estimation of pregnanetriol which is not stable when set free in hot acid solutions. Pregnanediol, however, is a very stable steroid and can be protected from prolonged contact with boiling dilute acid by a toluene overlay. For pregnanediol, acid hydrolysis is preferable to enzyme hydrolysis, as it is simpler, quicker, and can be more rigidly controlled, provided that it can be shown that hydrolysis is complete without destruction of the liberated alcohol and that other material set free by acid hydrolysis can be completely removed.

### 3. *Precipitation Procedures*

Precipitation procedures in the purification of steroid extracts have grave disadvantages. As applied in practice they depend on the fact that these steroids, which are soluble in organic solvents, are largely insoluble in water. The steroids can, therefore, be precipitated from water-miscible solvents by the addition of water. This would be an admirable procedure if there was a sharp critical point of dilution at which the steroid became insoluble. Pregnanediol becomes increasingly insoluble over a broad range of dilution with no final end point of complete insolubility. It is to some extent soluble in water. Alcoholic solutions of urinary extracts contain a great variety of steroids as well as other substances which are largely insoluble in water. Any set of conditions optimal for the precipitation of pregnanediol will to a greater or lesser extent overlap the precipitation range of these. Repeated precipitations greatly reduce this contamination but increase the loss of pregnanediol. It is also very difficult in a routine process to recover quantitatively from an aqueous medium a finely divided suspension of a steroid.

### 4. *Chromatography*

The introduction of chromatographic techniques has transformed steroid assays. These methods are eminently suitable to the purification of pregnanediol and pregnanetriol and should now replace older methods. The choice in fact is largely which type of chromatographic technique is most suited to the purposes of the assay and the facilities of the laboratory.

*a. Paper Partition Chromatography.* The particular advantage of paper chromatography in this field lies in its high degree of specificity. Properly used, paper chromatography can resolve very complex mixtures

of closely related compounds. It is therefore a reliable means of indicating the presence of a known compound or of comparing an unknown one with authentic steroids. As a tool in quantitative determination it is less useful. Only very small quantities of material can be analyzed. It is difficult to remove the pure material quantitatively from the paper, and material affecting subsequent color reactions is usually eluted with the steroid. It is a tedious and skilled technique not easily adaptable to large-scale use in routine laboratories.

*b. Column Partition Chromatography.* Column partition chromatography techniques share many of the advantages of paper chromatography and can in addition process larger amounts of material. They, like countercurrent methods, are eminently suitable for the preparation of larger amounts of material preliminary to its crystallization and identification by classic chemical methods. They are, however, essentially single-run techniques and are too elaborate and require too rigidly standardized conditions for routine assay purposes.

*c. Adsorption Chromatography.* Adsorption methods using columns of inert material such as alumina, are not as highly specific as paper chromatography but are eminently suitable for large-scale use in routine assays. Their powers of resolution can be greatly increased by repeated runs or by repeating the chromatography using derivatives of the steroid which is being assayed. Benzoates or acetates, for example, have quite different chromatographic properties from their parent alcohols. Very often impurities running close to the alcoholic steroid do not themselves esterify or else form esters which behave differently on the chromatogram from the esters of the steroid alcohol being assayed.

#### 5. Color Reactions

Simple weighing of the final residue is too unspecific and insensitive for most purposes. The sulfuric acid color reaction is very sensitive and under properly controlled conditions is an accurate means for measuring both steroids. The nature of the reaction and the final products formed are largely unknown. It is certain that a haphazard use of the reaction will not give good results. The factors controlling this reaction have been analyzed in a study by Klopper (1956). Fortunately pregnanetriol and pregnanediol are, among steroids, easily the most potent chromogens in sulfuric acid at the wavelengths examined. This confers a considerable degree of specificity on the reaction. Large amounts of, for example, cholesterol or of androstanediol would have to be present in the final residue to cause any overestimation of pregnanediol or pregnanetriol. The absorption peak most commonly used for pregnanediol is at 425–430  $m\mu$  and for pregnanetriol at 435–440  $m\mu$ . The absorption spectrum

of the latter in this region is particularly suitable for the Allen correction formula using readings at 400, 435, and 470  $m\mu$ .

## VII. Recommended Methods

### A. METHODS FOR THE ASSAY OF URINARY PREGNANEDIOL

#### 1. *A Technique Using Chromatography on Alumina Columns Published by Kloppe and His Associates (1955)*

In this method the alumina used is first deactivated and standardized in bulk. The alumina supplied by the manufacturers is spread out in closed flat-bottomed flasks with a container of water suspended above the alumina in order to ensure that the atmosphere is moisture saturated. The alumina is stirred from time to time. After 10–14 days it ceases to take up water from the atmosphere and the activity will then remain constant if it is stored in closed containers. The activity of the alumina is now measured against pregnanediol and pregnanediol diacetate. Model chromatographic columns are set up as used in the routine assay method. A few crystals of pregnanediol are dissolved in 10 ml. of toluene and poured onto a column made up in benzene. When all the solvent has percolated down the column it is eluted with successive 2-ml. lots of 0.8% ethanol in benzene until the first traces of pregnanediol can be detected in the residue when the eluant from the column is evaporated. This gives a measure of how many milliliters of 0.8% ethanol in benzene is required to convey the pregnanediol front from the top to the bottom of a 3-gm. column of the particular batch of deactivated alumina. A second column is now prepared as before. This time the column is eluted with 5 ml. less of 0.8% ethanol than is needed to take the pregnanediol front down the column. Thereafter the column is eluted with 2-ml. lots of 3% ethanol in benzene. With this stronger eluant, the pregnanediol, already carried well down the column by the previous elution with 0.8% ethanol in benzene, is rapidly removed from the column. Each 2-ml. fraction of stronger eluant after it has run down the column, is evaporated and the residue tested for pregnanediol with sulfuric acid, until no more pregnanediol is present. In this way it is possible to determine how much 3% ethanol in benzene is required to remove completely all pregnanediol from a column which has previously been eluted with a quantity of 0.8% ethanol in benzene which is just insufficient to remove any pregnanediol from the column. The same principle is applied to the standardization of the alumina against pregnanediol diacetate in the petroleum ether and benzene eluant system employed in the assay method. In this instance a prior elution with a weak eluant is not necessary and all that is required



is to determine how much benzene is required to remove all the pregnanediol diacetate when it has been put on the column dissolved in 25 ml. of petroleum ether.

Duplicate samples, one twentieth of the total 24-hour urine sample, are diluted to 150 ml. with water in a 500-ml. round-bottomed flask. Toluene (50 ml.) and a glass bead are added and the mixture is heated to boiling under a reflux condenser. Concentrated hydrochloric acid (15 ml.) is then added through the condenser and boiling continued for 10 minutes. The urine is run off from a separating funnel and re-extracted with a further 50 ml. of toluene. The combined toluene extracts are shaken with 25 ml. of 25% sodium chloride in 1 *N* sodium hydroxide solution. The aqueous layer is discarded and the toluene shaken for 10 minutes with 25 ml. of freshly prepared 4% potassium permanganate in 1 *N* sodium hydroxide solution. The toluene is washed with distilled water until all permanganate color is removed. The toluene is then filtered and distilled to a volume of approximately 10 ml.

A chromatography column is meantime prepared by pouring 3 gm. of deactivated standardized alumina into a chromatography tube filled with benzene. A convenient design for such a tube is one 12 cm. in length, with an internal diameter of 1 cm., a porosity 3 sintered glass disk in the base and a 50-ml. reservoir sealed onto the top. The volumes of eluants depend on the activity of the particular batch of alumina.

The toluene extract is applied to the column which is then eluted with 25 ml. of 0.8% ethanol in benzene. Thereafter it is eluted with 3% ethanol in benzene. This fraction contains all the pregnanediol and is collected in a test tube. The solvent is evaporated under nitrogen and the residue dissolved in 2 ml. of benzene. To this is added 2 ml. of acetyl chloride and the mixture is left at room temperature for an hour. Petroleum ether (25 ml.) is added, the solution is transferred to a separating funnel and washed successively with 25 ml. of water, 25 ml. of 8% sodium bicarbonate, and 25 ml. of water. The petroleum ether is then poured onto a 3-gm. alumina column made up in petroleum ether. This is then eluted with approximately 15 ml. of benzene, the exact volume being adjusted by standardization of a model column against pure pregnanediol diacetate as for the pregnanediol eluants.

The benzene fraction off the column is collected in a test tube and evaporated. Approximately 10 mg. of sodium sulfite and exactly 10 ml. of concentrated sulfuric acid is added to the residue. The tube is stoppered and left in a water bath at 25°C. overnight. The color density is read against a sulfite-sulfuric acid blank at 425  $\mu$ . The density is converted into milligrams of pregnanediol diacetate by reference to a calibration curve constructed from known amounts of pure pregnanediol.

diacetate. The true pregnanediol content is calculated by multiplying the pregnanediol diacetate value by 0.8, the ratio of the molecular weights. The authors claim that in 70 experiments in which the concentration of added pregnanediol was 0.5 mg. or higher per 24-hour urine sample the mean recovery was 94%. The standard deviation of replicate recoveries was 11% and the sensitivity of the method such that amounts as low as 0.5 mg./24-hour urine sample could be measured.

*2. A Technique Using Paper Chromatography Published by Eberlein and Bongiovanni (1958)*

A 10-ml. aliquot of the 24-hour urine sample is incubated for 12 to 15 hours at 37°C. with 1.0 ml. of acetate buffer (1.0 *M*, pH 4.5) and 3500 units of  $\beta$ -glucuronidase (Ketodase, Warner-Chilcott). The urine is extracted with 10 ml. of benzene which is then washed twice with 10 ml. of 1 *N* sodium hydroxide and twice with 10 ml. of water. The benzene is evaporated at 45°–50°C., under a stream of air.

Paper chromatography is done on  $9\frac{1}{8} \times 22\frac{1}{2}$  in. sheets of Whatman No. 2 filter paper. Strips 5.0 cm. long are cut from one narrow end leaving 5 application lanes each 1.0 cm. wide; the center of each lane is 4.6 cm. from the next. The urine extract is transferred to the application lane with methylene chloride and allowed to dry. In this way 4 extracts may be applied to the paper. Pure pregnanediol standard (15–25  $\mu$ g.) is applied to the fifth lane. The paper is then clipped onto a metal holder fitted with a trough into which the ends of the application lanes dip. To the trough is added 10 ml. of a mixture consisting of equal parts of methanol, ethyl acetate, and methylene chloride. In 10–15 minutes the solvent front carrying the extracts reaches a starting line marked on the paper 9 cm. from the end. The remaining solvent is aspirated and the paper is dried under a hair dryer. An ascending chromatogram is now developed. The tank of the chromatography apparatus is filled with a stationary phase consisting of a mixture of 400 ml. of methanol and 100 ml. of water equilibrated with a mobile phase of 225 ml. of isooctane with 275 ml. of toluene. The paper holder is inserted and the apparatus left to equilibrate at room temperature overnight. In the morning the mobile phase is added to the trough on the paper holder and development at room temperature allowed to proceed for 6 hours. The paper is then removed and dried. Pencil lines are drawn 4.6, 9.2, 13.8, and 18.4 cm. from the left edge. The left lane containing the standard is cut away and dipped in 5 ml. of a solution of 4 gm. of phosphomolybdic acid in 100 ml. of ethanol. It is blotted and heated at 80°C. for 5 minutes. The solvent front is found to be 34–36 cm. from the starting line and the standard, staining blue, to have an  $R_f$  value of 0.5. The standard strip is then fitted

back against the remaining chromatogram and horizontal lines drawn across 1.5 cm. above and below the center of the standard spot. The rectangles thus outlined on each chromatogram are cut out and snipped into small pieces which are soaked for 3 hours in 5 ml. of methanol. This methanol is decanted and the soaking repeated in two lots of 5 ml. of fresh methanol for 15 minutes on each occasion. The methanol is evaporated under a stream of air. A chromatographic column of 3 gm. of silica gel made up in 1% ethanol in methylene chloride is washed with 10 ml. of this solvent and the eluate from the paper transferred to the column with five 2-ml. portions of 1% ethanol in methylene chloride. The column is then washed with 20 ml. of 2% ethanol in methylene chloride and the pregnanediol eluted with 20 ml. of 7.5% ethanol in methylene chloride. The solvent is evaporated and 2 ml. of bisulfite-sulfuric mixture (50–60 gm. sodium bisulfite in 200 ml. of concentrated sulfuric acid) is added to the residue. This is heated in a boiling water bath for 4 minutes, allowed to cool for 20 minutes, and then read at 390, 425, and 460  $m\mu$  against a sulfite-acid blank. The Allen formula is used to obtain the corrected density at 425  $m\mu$ , which is translated into micrograms of pregnanediol by reference to the reading of the standard.

The reliability data published by Eberlein and Bongiovanni (1958) suggest that this method is comparable to that published by Kloppe *et al.* (1955). The clinical results published for both methods appear to be of much the same order.

#### B. METHODS FOR THE ASSAY OF URINARY PREGNANETRIOL

##### 1. *A Technique Using Chromatography on Alumina Columns Devised by Stern (1957)*

This method is based on the pregnanediol determination technique of Kloppe *et al.* (1955). The preparation of the columns, inactivation and standardization of the alumina, and the composition of the eluants is therefore as described in Section VII, A, 1.

Duplicate aliquots, one eightieth of the total 24-hour urine sample, are made up to 25 ml. with distilled water and 12.5 gm. of ammonium sulfate is added. The solution is then extracted 3 times with 12.5 ml. of a mixture of 1 part ethanol to 3 parts ether. The ethanol-ether extractions are pooled and evaporated. To the residue is added 15 ml. of 0.1 *N* ammonium acetate buffer (pH 4.5) and 15,000–30,000 Fishman units of  $\beta$ -glucuronidase. The mixture is incubated at 37°C. for 4 hours. It is then extracted 3 times with 10 ml. of benzene. The combined benzene extracts are washed 3 times with 8 ml. of 1 *N* sodium hydroxide solution containing 25% sodium chloride followed by 3 washes with 8 ml. of

water. The benzene extract is evaporated to 5–10 ml. and poured onto a 3-gm. alumina column made up as for pregnanediol in Section VII, A, 1. The column is eluted with 25 ml. of 0.8% ethanol in benzene. The pregnanediol is then eluted with 12 ml. of 3% ethanol in benzene. The pregnanetriol is next eluted with 12 ml. of 10% ethanol in benzene. The pregnanediol may now be estimated by putting it through the remainder of the procedure of Klopper *et al.* The solvent fraction containing the pregnanetriol is evaporated and the residue dried in a desiccator over calcium chloride. Three milliliters of concentrated sulfuric acid is added to the dried residue and the solution left overnight at room temperature, a standard of approximately 0.02 mg. of pure pregnanetriol being similarly treated. The densities are read in a spectrophotometer at 400, 435, and 470  $m\mu$ . The corrected density at 435  $m\mu$ , is then worked out by the Allen formula (1950) and the pregnanetriol equivalent calculated by reference to the corrected density of the standard. When pure pregnanetriol was added to urine, Stern (1957) obtained recoveries ranging from 71 to 103% in 12 experiments. The final residue from urine gave results very similar to that from the pure steroid when the infrared spectrum and the visible light spectrum of a sulfuric acid solution were examined. The countercurrent behavior also corresponded to that of pure pregnanetriol.

*2. A Technique Using Chromatography on Alumina Columns Devised by Bongiovanni and Eberlein (1958)*

The principles of this method are the same as those of Stern's method but, unlike the latter, it is not geared to the simultaneous estimation of pregnanediol and may, therefore, be preferable in assays of pregnanetriol only.

A 10-ml. sample of the 24-hour urine sample is incubated with 1 ml. of 1 *M* acetate buffer and 5000 units of glucuronidase at 37°C. for 24 hours. The urine is then extracted 3 times with 10 ml. of dichloromethane. The pooled extracts are washed twice with 10 ml. of 0.1 *M* sodium hydroxide and twice with 10 ml. of water. The solvent is evaporated and the residue transferred to an alumina column with two lots of benzene, 10 ml., followed by a 20-ml. rinse. The column is made up in benzene and has 7 cm. of alumina in a glass tube 4–6 mm. in diameter. The column is then eluted with 40 ml. of 2% ethanol in benzene and the pregnanetriol brought down with 30 ml. of 6% ethyl alcohol in benzene. The solvent is evaporated and the color developed with 10 ml. of concentrated sulfuric acid at room temperature for 30 to 120 minutes. The densities are read at 410, 440, and 470  $m\mu$  together with the density of a standard of 10 to 40  $\mu$ g. of pregnanetriol. The pregnanetriol content of the residue

is then calculated with reference to the standard, the Allen correction formula being employed.

These workers tried the effect of a preliminary elimination of ketonic compounds by Girard separation but did not find that the introduction of this step materially altered the results. They also tried methods other than the sulfuric acid color reaction for reading the final residue. They used chromic acid oxidation and estimation as 17-oxosteroids, periodate oxidation with estimation of the acetaldehyde liberated (Cox, 1952), the development of a modified Kober color, or fluorescence. None of these were found to be a significant improvement on the sulfuric acid reaction. It is difficult to deduce the true sensitivity of this method but from the data published by the authors it is unlikely that it can measure quantities below 0.5 mg./24-hour urine sample. At somewhat higher levels the recoveries ranged from 86–92%. The average difference between duplicates was 1.3%. Specificity studies of residues by paper chromatography and infrared analysis were satisfactory.

The best features of the methods of Stern and of Bongiovanni and Eberlein have recently been combined by Fotherby (1960) and it is likely that this method will be very useful when details become available.

### VIII. The Clinical Applications of the Assay of Pregnanediol and Pregnanetriol

#### A. OVARIAN STUDIES

##### 1. *Studies on Pregnanediol Excretion in the Normal Menstrual Cycle*

Since 1936 every year has produced new studies on this subject. The pattern of excretion and its relationship to various physiological events in the cycle is clearly established. The earlier studies were done by methods too insensitive to detect the "adrenal" pregnanediol in the proliferative phase and too inaccurate and unspecific to give reliable figures at any stage. Later studies have brought out the finer details, showing that there is slight residual activity in the corpus luteum of the previous cycle for the first few days after the onset of bleeding and that excretion then levels off at roughly 1 mg. per day. There is a slight preovulatory rise; the levels attained in the luteal phase may reflect the growth of the corpus luteum and the onset of bleeding is preceded by a sharp fall in pregnanediol output. Figures 4, 5, and 6 show three basic patterns of pregnanediol output from a study by Klopper (1957). Figure 4 shows the pattern of excretion shown by a sexually mature adult nulliparous woman. The luteal phase is clearly defined reaching a maximum excretion of 3 to 5 mg. pregnanediol per 24 hours. Figure 5 shows the juvenile

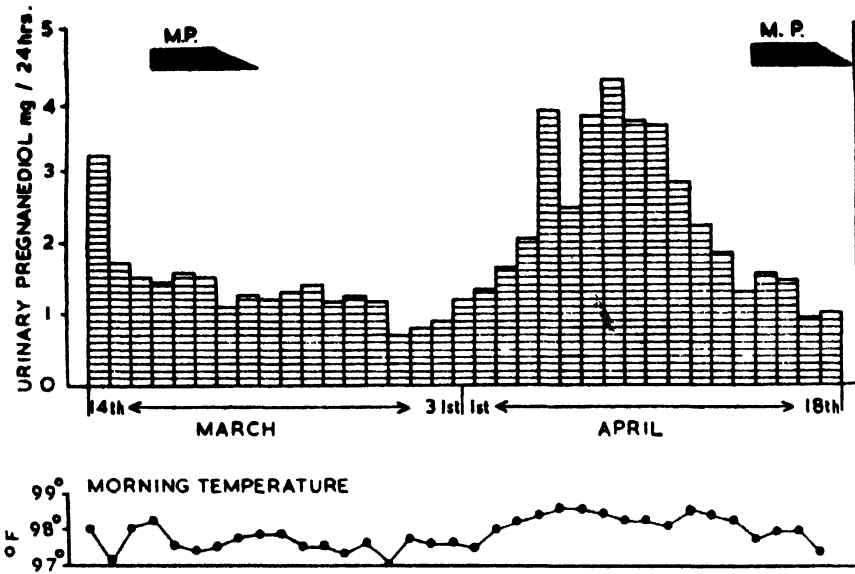


FIG. 4. Urinary excretion of pregnanediol in the menstrual cycle. Sexually mature nulliparous pattern. Basal temperature readings are also shown. M.P. = menstrual period. From Klopper (1957).

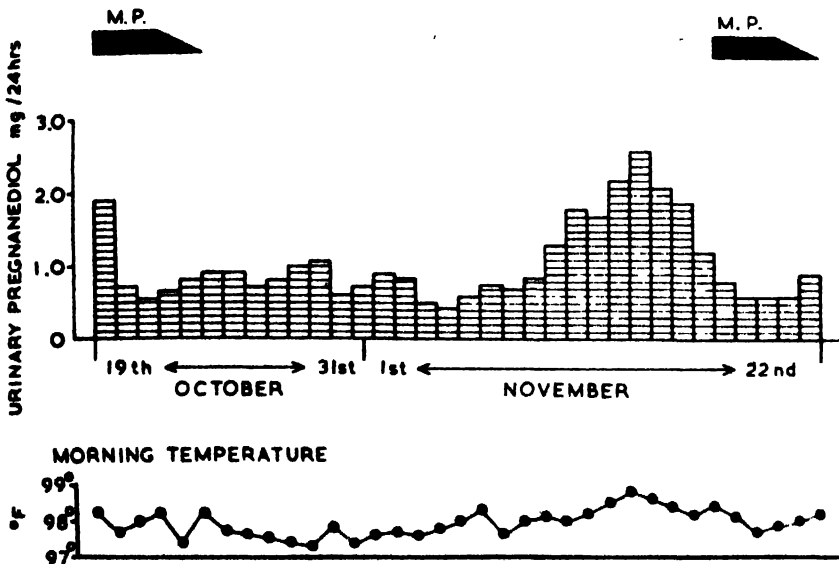


FIG. 5. Urinary excretion of pregnanediol in the menstrual cycle. Juvenile nulliparous pattern. Basal temperature readings are also shown. M.P. = menstrual period. From Klopper (1957).

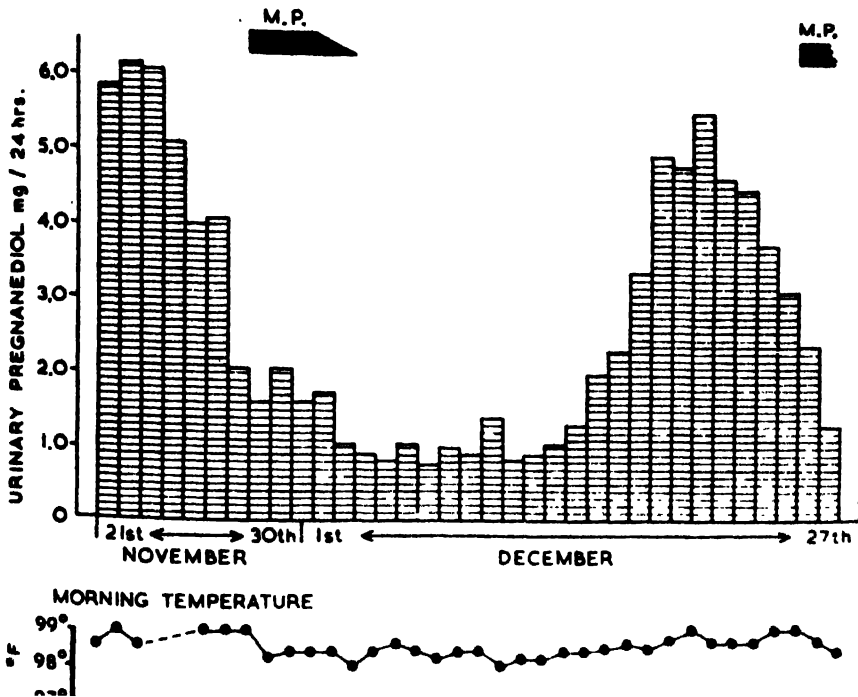


FIG. 6. Urinary excretion of pregnanediol in the menstrual cycle. Adult parous pattern. Basal temperature readings are also shown. M.P. = menstrual period. From Klopper (1957).

pattern found in a young woman of 15 years. Here the luteal phase can barely be distinguished, rising to a maximum of 2 to 3 mg./24 hours. Figure 6 shows the florid development of the corpus luteum in an adult parous woman, pregnanediol excretion rising to above 6 mg. in the luteal maximum.

## 2. Fertility Studies

Assays of urinary pregnanediol may be of help in determining the cause of infertility. They are a convenient way of deciding whether a corpus luteum is present and how actively it is functioning. If the three basic patterns described by Klopper are real entities, the poorly developed luteal phase of the juvenile type may account for infertility and may be amenable to treatment by progestogens.

## 3. Abnormalities of Menstruation

In conditions such as anovular bleeding, secondary amenorrhea, persistent corpus luteum, or irregular shedding of the endometrium, assays

of urinary pregnanediol may be of value in establishing the diagnosis or assessing the results of treatment. In the rarer masculinizing syndromes such as adrenal hyperplasia, idiopathic or hereditary hypertrichosis, arrhenoblastoma, or Stein-Leventhal syndrome assays of urinary pregnanediol and pregnanetriol are important. In adrenal hyperplasia a raised pregnanetriol output is almost invariably present. It has been shown that the Stein-Leventhal syndrome is not associated with a raised pregnanediol output. Reliable pregnanetriol assays may prove of great value in establishing the identity of the ovarian androgen in this disease.

## B. PLACENTAL STUDIES

### 1. Abortion

A great many studies have been published concerning pregnanediol output in recurrent and threatened abortion. Modern assay methods have confirmed the value of urinary pregnanediol levels in prognosis. Not all abortions, however, are of endocrine origin. Few of those due to the considerable variety of other causes show a fall in pregnanediol output sufficiently early to be of any value. In evaluating the results of any endocrine therapy of abortion pregnanediol assays are probably the most valuable single criterion.

### 2. Placental Function in Late Pregnancy

Several studies have been published using pregnanediol output as an index of placental function in conditions such as postmaturity and toxemia. In this respect the work of Shearman (1959) has been most useful. It is apparent that when the mean outputs of groups of patients are considered, valuable generalizations about the results of disease may be made, but the natural variation from day to day in normal output and the variation from person to person is too large for secure judgment in the single individual. In this field, too, more than elsewhere, it is evident that a single 24-hour value is inadequate and judgments have to be based on a series of assays.

## C. ADRENAL STUDIES

Klopper *et al.* (1957) suggested the use of pregnanediol assays as a measure of adrenal function. This idea has not been widely used probably because urinary pregnanediol output reflects the available steroid building material in the adrenal rather than the levels of a specific hormone



with defined functions. Pregnanediol assays may be of more specific value in the study of such conditions as hirsuties of adrenal origin. In the field of adrenal function, generally pregnanetriol assays are more useful and likely to be of increasing importance in the future when the quantitative relationship of pregnanetriol to its precursors is defined and the role of these precursors in adrenal physiology is better understood.

### **IX. Future Developments in the Assay of Pregnanediol and Pregnanetriol**

As a research tool the more obvious uses of pregnanediol assay have been fully exploited in the last twenty years. The same does not apply to pregnanetriol where urinary assays may break new ground. The scope of both assays in clinical routine has been defined. At least in the case of pregnanediol the assay is probably sufficiently useful in clinical practice to justify its establishment in any sizable center.

It has not been clearly realized that these urinary steroid assays have certain grave disadvantages in practice. They may in fact give a deceptive picture of the production of the original active hormone. To begin with the gut is a possible unmeasured source of steroid loss. A rise or fall in urinary steroid output may reflect corresponding changes in fecal loss rather than changes in the production of the original hormone. Fecal excretion is unlikely, however, to be a major factor contributing to the day-to-day variation in pregnanediol output. It has already been noted that the percentage conversion of injected progesterone to urinary pregnanediol excreted falls in a narrow range. It is also unlikely that the comparatively subtle patterns of pregnanediol excretion in such conditions as the normal menstrual cycle or in operative stress would repeat themselves so consistently if the excretion levels were liable to be much influenced by aberrant gut loss of pregnanediol. Secondly the urinary metabolite measured is only one of a large number of end products of the active hormone. A rise or fall in the output of a particular metabolite may mean only that less or more of other metabolites were excreted. Other processes also intervene between the production of the active hormone and the excretion of its inert metabolite, notably the role of the liver in conjugation or of the kidney in excretion. There is no evidence that these have any profound effect in producing variations of metabolite excretion. The causes of physiological variation of steroid excretion which have been mentioned make it necessary to view with reserve the use of assays in the control of the day-to-day therapy of the

individual case and with suspicion any deductions based on single observations. It is in the retrospective survey of serial determinations that such steroid assays are of most use in diagnosis and treatment.

In purely methodological terms there would seem to be little need to seek for further refinements making the present assay techniques more accurate, at least as far as pregnanediol is concerned. Particularly as applied to pregnancy urines the accuracy of the laboratory processes has outstripped the accuracy of the physiological control of the subject. Much of the future development in this field depends on a clearer understanding and more rigid standardization of such factors as nutritional state, fluid balance, and body build. The most promising research line consists of serial observations on subjects in research units where physiological variables can be standardized as far as possible.

It is also likely that in the future more emphasis will be placed on hormone measurements in blood (see Chapter 3). There are, however, cogent reasons for believing that in some respects blood assays may prove inferior to urinary determinations. The primary hormone, progesterone, has such a short life in the circulation that measurements of it in peripheral blood have very little value. Determinations of biologically stable metabolites like pregnanediol in blood are the equivalent of a single snapshot of a complicated moving process. They represent only the concentration at the moment the blood was withdrawn while urine determinations are at least a summation of the events of 24 hours. Blood volumes vary considerably from subject to subject, particularly in pregnancy and may change rapidly from time to time in the same person. Any simple concentration figure may therefore have more relevance to the circulatory volume than to the hormone production of the placenta or the ovaries.

There is a limited scope for the development of more sensitive methods. Existing methods are adequate for present clinical purposes. Research interests are centering more on the mode and site of action of hormones and on special studies such as the excretion of minute amounts of steroid hormone metabolites after extensive endocrine ablative surgery for the treatment of recurrent breast cancer. For such purposes more sensitive methods for hormone measurements in blood, tissues, and urine will need to be evolved.

The future development of urinary assays in this field lies not so much in improvement of methods in the laboratory as it does in using present methods more rationally. The range of values for normal and pathological states needs to be more clearly defined. The physiological state of the subject on whom observations are made needs to be more categorized, more rigidly controlled, and consciously manipulated. Evi-

dently the overlap between the values found in normal and diseased states is such that snap judgments on individual cases are unwise and the best we can hope for are useful generalizations about the trend in any particular condition.

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## Chapter 5

# Pregnanetriolone, an Abnormal Urinary Steroid\*

MICHAEL FINKELSTEIN

I. Introduction	169
II. Historical	170
III. Methods for the Assay of Pregnanetriolone and Related Steroids in Urine	171
A. General Considerations	171
B. Analytical Procedure	172
IV. Preparation of the Urinary Extracts (Methods I and II)	
According to Cox and Finkelstein (1957)	173
A. Collection of Urine and Hydrolysis	173
B. Extraction	173
C. Paper Chromatography (Methods I and II)	173
V. Method Ia	176
VI. Methods I and Ia: Quantitative Estimation by Fluorometry	177
A. Equipment	177
B. Procedure	177
VII. Method II: Quantitative Estimation through the Determination of Acetaldehyde ("Acetaldehydogenic" Steroids)	179
Procedure	179
VIII. Other Methods	180
IX. Occurrence of Pregnanetriolone	180
X. Cases Where Pregnanetriolone Was Not Found	180
A. Normal Persons	180
B. Stimulation with ACTH	181
C. Pregnancy	181
D. Hirsutism	181
E. Hermaphroditism and Pseudohermaphroditism Due to Extra-adrenal Factors	181
F. Adrenal Adenomas and Carcinoma	182
XI. Conditions in Which Pregnanetriolone Is Excreted	186
A. Congenital Adrenal Hyperplasia	186
B. Cushing's Syndrome Due to Bilateral Adrenal Hyperplasia	189
C. Cushing's Syndrome with No Evidence of Adrenal Hyperplasia	190
D. Postnatal Virilization Syndrome and Stein-Leventhal Syndrome	191
XII. Biosynthesis of Pregnanetriolone	193
References	196

## I. Introduction

Certain changes in the metabolism of adrenal steroids may lead to a variety of pathological conditions. These changes may be due to several

\* This article is dedicated to Professor Bernhard Zondek whose long-standing friendship and guidance has been a great stimulus in carrying out this study.

factors either intrinsic or extrinsic to the adrenal cortex. Whatever the immediate cause of the abnormality may be, it is customary to refer to it in terms of hypo- or hyperactivity of the adrenal cortex. However, these clinical *hypo-* or *hyper-*states cannot always be detected by laboratory analytical methods or by histological examinations. Numerous cases of Cushing's syndrome have been described without histological abnormality of the adrenals and without a substantial increase in the excretion of the adrenal steroids (Soffer *et al.*, 1955). Similarly, in many cases of pronounced virilization normal excretion of 17-ketosteroids has been noted. Evidently in such cases, the routine methods of analysis measuring a group of compounds are not sufficiently specific to distinguish the causative factor. On the other hand methods which estimate the individual steroids have a better chance to pinpoint either the active compound directly responsible for the pathological condition, or an inactive metabolite which may reflect the events leading to the abnormality. Aldosterone, testosterone, and cortisol, all being active hormones, may serve as examples of the first alternative. On the other hand estimating pregnanediol, pregnanetriol, tetrahydrocortisol, etc., may indicate the fate of their respective precursors.

This chapter will be devoted to the discussion of  $5\beta$ -pregnanc- $3\alpha,17\alpha,20\alpha$ -triol-11-one (pregnanetriolone), an inactive urinary metabolite, belonging to the second class. The occurrence of pregnanetriolone, the significance of its estimation as an aid in differential diagnosis, the methods of estimation, and the metabolic pathways leading to the excretion of pregnanetriolone and related steroids will be reviewed.

## II. Historical

In the early 1950's Zondek and Finkelstein (1952) showed that in cases of female pseudohermaphroditism due to congenital adrenal hyperplasia a urinary neutral substance is excreted, which becomes fluorescent on reacting with hot phosphoric acid. This material was subsequently isolated by Finkelstein *et al.* (1953) from the urine of two patients with congenital adrenal hyperplasia and identified as pregnanetriolone. Fukushima and Gallagher (1957) confirmed the finding by isolating this compound in other cases of congenital adrenal hyperplasia. In addition, the latter authors isolated a closely related steroid,  $5\beta$ -pregnanc- $3\alpha,11\beta,17\alpha,20\alpha$ -tetrol (pregnanetetrol), from the urine of the same patients. Since then the compound has become of considerable interest, because of the unique quality of being excreted in measurable quantities only in the urine of patients with a certain form of adrenal dysfunction. Several

terms have been used for pregnanetriolone, such as 11-keto-pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol and trihydroxy-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -pregnane-11-one. Fieser and Fieser (1959) used the name 5 $\beta$ -pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol-11-one, and the present author prefers this term, especially since it is easily abbreviated as "pregnanetriolone."

### III. Methods for the Assay of Pregnanetriolone and Related Steroids in Urine

#### A. GENERAL CONSIDERATIONS

The validity of an analytical method for the determination of a single compound in a mixture, in which the compound constitutes a small fraction, depends on whether the compound can be separated and estimated in such a way that it will not be influenced by the other constituents of the mixture. This is extremely important in the case of urinary steroids in general since they usually are excreted in a concentration which is very low in relation to the interfering substances. Moreover, many of the urinary steroids have similar properties and with insufficient care one can be easily mistaken for another. In principle the separation may be achieved by physicochemical means such as chromatography or by the use of a precise detection method, which is specific for a single compound. The last alternative does not seem practical unless sufficient purification has been achieved to eliminate most of the interfering substances. Thus a combination of an effective separation procedure with a precise and sensitive method for the final determination offers, at the present time, the best solution for the estimation of certain urinary steroids. The accuracy of such determinations may be of diagnostic importance, especially in diseases where abnormal excretion of one or more steroids is characteristic. The case of pregnanetriolone may be a "classic" example.

The first indication of the presence of pregnanetriolone in urine was obtained in two cases of female pseudohermaphroditism due to adrenal hyperplasia, following the observation that the neutral fraction produces an intense fluorescence when heated with 85% phosphoric acid. Guided by this reaction the fluorogenic compound was isolated and identified as pregnanetriolone (Finkelstein *et al.*, 1953). However, urine from normal individuals also contains neutral compounds which produce fluorescence with phosphoric acid, though considerably less than in the urinary extracts of cases of adrenal hyperplasia. On the other hand in urine from late pregnancy cases and especially from some cases of adrenal cancer

the concentration of the fluorogenic material was comparable to that associated with adrenal hyperplasia patients (Zondek and Finkelstein, 1952; Finkelstein, 1953). In all these cases, other than adrenal hyperplasia, isolation of pregnanetriolone was not achieved (Goldberg, 1957; Finkelstein, 1959). This apparent discrepancy was explained only after a specific method for the estimation of pregnanetriolone was developed (see Analytical Procedure, Section III, B). Analysis of urines with this new method has shown that the excretion of pregnanetriolone is essentially confined to cases of adrenal hyperplasia, and that the fluorescence obtained on the crude neutral fraction from urine of other patients was due to compounds other than pregnanetriolone. This selective excretion of a single compound could serve as a diagnostic test in the differentiation between adrenal hyperplasia and other conditions leading to similar clinical symptoms, provided that supporting evidence could be obtained for a sufficiently large number of cases in each group. Accordingly, after the development of suitable methods, a systematic search for pregnanetriolone was undertaken in urine of normal subjects, and also in such conditions where its presence was suspected by clinical diagnosis or by the intensity of fluorescence of the crude extracts.

#### B. ANALYTICAL PROCEDURE

Two methods have been developed for the quantitative estimation of pregnanetriolone and related steroids. In both of them the compounds to be analyzed are first isolated in a high degree of purity and then submitted to two different procedures.

In method I pregnanetriolone is estimated fluorometrically (Finkelstein, 1959). The sensitivity is about 50  $\mu\text{g.}/24\text{-hour}$  urine sample. In addition, pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ -triol-11-one, pregnanetriol, and pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ -triol may be estimated. In method Ia only pregnanetriolone (20 $\alpha$ -) and its 20 $\beta$ -epimer may be estimated. The sensitivity is increased to about 2  $\mu\text{g.}/24\text{-hour}$  urine sample.

In method II (Cox, 1959), the procedure consists of oxidizing pregnanetriolone with periodic acid and estimating the formed acetaldehyde. In principle, all C<sub>21</sub> deoxysteroids hydroxylated at both C-17 and C-20 positions may be estimated by this method. Methods I and II were found to be equally sensitive. Comparative results obtained by both methods agreed well with each other and the choice between them should be made on the basis of the available setup. Method I was essential for the isolation of pregnanetriolone from urine of patients with congenital adrenal hyperplasia and method II for the isolation of pregnanetriol from normal human urine (Finkelstein *et al.*, 1953; Cox and Marrian, 1953).



#### IV. Preparation of the Urinary Extracts (Methods I and II) According to Cox and Finkelstein (1957)

##### A. COLLECTION OF URINE AND HYDROLYSIS

A 24-hour urine sample collected over 2 ml. of chloroform is obtained and kept refrigerated until assayed. The volume is measured. One fifth of the urine is withdrawn and the pH is adjusted to 4.6 with glacial acetic acid, and 2 ml. of 2 *M* acetate buffer at pH 4.6. The sample is warmed in an incubator at 37°C. for 1 hour and 50 units of  $\beta$ -glucuronidase per milliliter of urine are added (Ketodase or a preparation obtained from the mollusc *Cellana tramoserica* has been used). The samples are allowed to stand at 37°C for 48 hours.

##### B. EXTRACTION

After hydrolysis, the urine is cooled to room temperature and extracted twice with equal volumes of freshly distilled chloroform. The pooled chloroform extract is washed with 0.1 volume of *N* sodium hydroxide and the alkali is drained off as thoroughly as possible. The chloroform is then washed successively with 20 ml. saturated sodium bicarbonate and twice with 20 ml. distilled water. The washed chloroform solution is dried over anhydrous sodium sulfate and filtered into a round-bottomed flask for evaporation *in vacuo*. The residue is dissolved with slight warming in about 5 ml. methanol. The solution is equally divided into four portions each of which is equivalent to 0.05 of the original 24-hour urine sample. The contents of the tubes are evaporated to dryness under nitrogen and are ready for chromatography to separate the individual compounds.

##### C. PAPER CHROMATOGRAPHY (METHODS I AND II)

Whatman No. 1 paper "for chromatography" is used. In method I the paper is purified by percolating benzene through it in a chromatography tank for 24 hours. In method II the paper may be used without purification. For each urine sample a chromatogram strip is cut with six tongues 1.0 cm. wide, 50 cm. long, and separated by 0.5 cm. from one another. The separation of the steroids is done on two consecutive chromatograms. For the first chromatogram the benzene-formamide system is used, and for the second either chloroform-formamide or ethylene dichloride-formamide (Zaffaroni and Burton, 1951). In both systems the paper is impregnated with methanol: formamide (1:1). The chromatog-

raphy is carried out at  $27^\circ \pm 1$ . In the first chromatogram the urinary extracts are applied to the four middle strips, and the appropriate standards are applied to two strips on each end of the chromatogram, and to one of the middle strips carrying the extract. After an overnight run (about 12–13 hours) the chromatogram is dried in an oven (temperature  $80^\circ$ – $90^\circ$ ). Strips carrying the standards, the standards plus extract, and one of the extracts alone, are cut off and developed for the detection of the specific compound.

The detection is done by dipping each strip in a solution of 70% phosphoric acid. The excess of the acid is removed by blotting the strip thoroughly between filter paper. The strips are put immediately on a preheated enamel tray in an oven (initial temperature  $85^\circ$ – $87^\circ$ ) for 10 minutes. Care should be taken that the temperature remains constant so no charring of the paper occurs. The tray is taken out and quickly cooled on ice cubes. Without delay, the strips are examined on the tray under an intense ultraviolet (UV) lamp with a filter passing light mainly of 365 m $\mu$ . The standard compounds separate in the following order from the origin: cortisol; pregnanetetrol; pregnanetriolone;  $3\alpha,17\alpha,20\beta$ -trihydroxy- $5\beta$ -pregnan-11-one; pregn-5-ene- $3\beta,17\alpha,20\alpha$ -triol; pregnanetriol;  $5\alpha$ -pregnane- $3\alpha,17\alpha,20\alpha$ -triol; and  $5\beta$ -pregnane- $3\alpha,17\alpha,20\beta$ -triol. The fluorescent colors of the compounds in the UV and their colors in daylight are described in Table I. The fluorescence is usually more intense than

TABLE I

FLUORESCENCE AND COLOR OF STEROIDS IN THE 70% PHOSPHORIC ACID REACTION

Steroid	Color in daylight	Fluorescence in UV	Sensitivity ( $\mu\text{g}/\text{cm}^2$ ) and authors <sup>a</sup>
Cortisol	Yellow	Brilliant yellow	0.3–0.6 (M.F.)
$5\beta$ -Pregnane- $3\alpha,11\beta,17\alpha,20\alpha$ -tetrol	Purple	Pink	1–2 (R.I.C.); 0.3–0.6 (M.F.)
$3\alpha,17\alpha,20\alpha$ -Trihydroxy- $5\beta$ -pregnan-11-one	None	Blue-violet	0.3–0.6 (R.I.C.); 0.7–1.5 (M.F.)
$3\alpha,17\alpha,20\beta$ -Trihydroxy- $5\beta$ -pregnan-11-one	None	Blue-violet	0.7–1.5 (M.F.)
$5\beta$ -Pregn-5-ene- $3\beta,17\alpha,20\alpha$ -triol	Purple	Yellow	2–4 (R.I.C.)
$5\beta$ -Pregnane- $3\alpha,17\alpha,20\alpha$ -triol	Purple	Pink	1–2 (R.I.C.); 7–15 (M.F.)
$5\alpha$ -Pregnane- $3\alpha,17\alpha,20\alpha$ -triol	Purple	Pink-brown	1–2 (R.I.C.)
$5\beta$ -Pregnane- $3\alpha,17\alpha,20\beta$ -triol	Purple	Violet-pink	1–2 (R.I.C.)

<sup>a</sup> R.I.C.—R. I. Cox (1959); M.F.—Present author.

the color in daylight. The paper strips with the urinary extract and with the extract and added standards are compared with the strips carrying the pure standard steroids. The purpose of the strip with the standards added to the extract is to check whether urinary contamination interferes with the development of fluorescence or color by the respective steroids. If no interference is seen a semiquantitative estimation can be made at this stage by comparing the fluorescence of the various regions of the chromatogram of the unknown sample with the strips on which the crystalline steroids were chromatographed.

The two strips with the extract, which have not been dipped in phosphoric acid, are cut according to the position of the steroids found in the developed strips with the standards and the extract. An extra margin of 0.5 to 1 cm. is allowed at each edge of the spot. Each piece of paper is cut into small pieces and extracted separately by soaking in a few milliliters of methanol. The methanol is decanted into a tube and the paper is washed twice with additional portions of methanol. All the washings are combined in the tube and evaporated to dryness under a stream of nitrogen. For chromatography the residue is dissolved in a few drops of methanol with a little warming, while rotating the tube. The second chromatogram is carried out in the chloroform (or ethylene dichloride)

formamide system. The separation of pregnanetriol and pregnanetriolone is shown in Fig. 1. Eluates of each region corresponding to the appropriate standard are chromatographed individually on a strip, on each side of which a strip with the standard is run. This is done in duplicate. One strip is used for the detection of the compound by the phosphoric acid method described above, and the second one serves for the quantitative estimation. The average time for the solvent front to reach the bottom of the chromatogram is about 3 hours. In most cases these two chromatograms suffice to obtain spots indistinguishable from the standard solutions. If contamination is present it is recommended to rechromatograph the eluates in the first system (e.g., benzene-formamide) and prolong the time of chromatography.

For the quantitative estimation with method I (see below) the regions of the chromatograms corresponding to the standards are eluted in the same fashion as described above. They are collected in small tubes, evaporated to dryness, and then are ready for quantitative estimation. For method II the regions are just cut out and the estimation is done directly on them.

These descriptions of chromatographic procedures should serve only as an example, since in many other systems an equally efficient separation may be obtained. Care should be taken however *not to use* systems which utilize propylene glycol for impregnation if method II is being

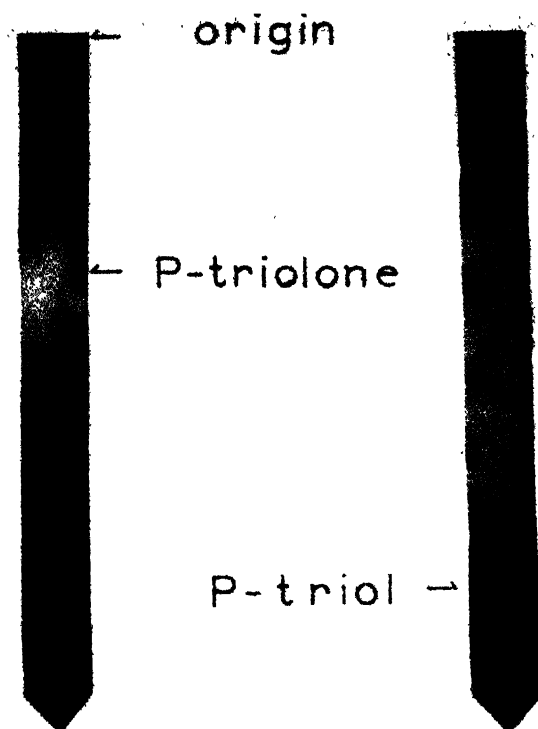


FIG. 1. Separation of pregnanetriolone (P-triolone) and pregnanetriol (P-triol) by paper chromatography in the system chloroform-formamide. The strip on the left side, where pregnanetriolone is visible was photographed in UV light, with a Wratten-2B filter in front of the camera. On the right side, where pregnanetriol is visible, the same strip is photographed in daylight.

used for quantitative estimation, because glycol yields acetaldehyde on oxidation with periodic acid.

#### V. Method Ia (Finkelstein, 1959)

This method of extracting and purification of the extract is used when a greater sensitivity is required, e.g., where it is especially important to estimate pregnanetriolone in concentrations below 50  $\mu\text{g.}/24\text{-hour}$  urine sample. It is essentially similar to method I, but the entire 24-hour collection is used for hydrolysis and extraction. The concentration of the enzyme for hydrolysis is proportionally increased as is also the volume of chloroform for the extraction. After washing the chloroform extract with 1 *N* sodium hydroxide, sodium bicarbonate, and water (the

volume should be adjusted to the proportions described for methods I and II), the solvent is evaporated *in vacuo*. The dry extract is redissolved in a minimal amount of ethanol and then diluted with 50 ml. benzene and 50 ml. petroleum ether. This solution is extracted twice with equal volumes of water and then re-extracted from water into ether. The ether is evaporated *in vacuo* and the residue dissolved in 20 ml. methanol which is then diluted with water to 50%. The 50% methanol solution is washed once with an equal volume of carbon tetrachloride, and the layers are carefully separated by centrifugation. The clear methanolic phase is drained off and evaporated *in vacuo*. The residue is dissolved in a minimal quantity of absolute methanol and applied to paper for chromatography, which is performed in a similar way as described above, but strips 2 cm. wide are used. In this modification pregnanetriol disappears almost completely, and therefore the method is only valid for estimating pregnanetriolone and its 20 $\beta$ -epimer. The limit of sensitivity is about 2  $\mu$ g./24-hour urine sample. In experiments where 2  $\mu$ g. were added to a 24-hour normal urine sample (not containing pregnanetriolone) 80% was recovered.

## VI. Methods I and Ia: Quantitative Estimation by Fluorometry (Zondek and Finkelstein 1952; Finkelstein and Goldberg, 1957)

The following steroids have been quantitatively estimated; cortisol; pregnanetriolone; 3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ -trihydroxy-5 $\beta$ -pregnan-11-one, and pregnanetriol. The fluorometric procedure is similar to the one developed for estimating urinary estrogens (Finkelstein, 1952).

### A. EQUIPMENT

(a) Lighttight hot water bath with tube rack equipped with arms for intermittent manual shaking. (b) Reaction tubes for developing fluorescence: Pyrex tubes (5-ml. capacity) fitted with male ground joints and female glass stoppers. (c) Fluorometer: Farrand, model A provided with interference and glass filters. (d) Phosphoric acid: "Baker Analyzed" 86% (J. T. Baker Company, Phillipsburg, New Jersey).

### B. PROCEDURE

The dry residues of the eluates of the zones corresponding either to cortisol, pregnanetriolone (or 20 $\beta$ -stereoisomer), or pregnanetriol, are dissolved in accurately measured double-distilled 96% ethanol. Aliquots

of these solutions containing approximately 0.1–0.5  $\mu\text{g}$ . of any of the above compounds are transferred to reaction tubes (see Equipment) and evaporated to dryness in an oven at  $100^\circ$ . The approximation of the concentration can be made by comparing the fluorescent colors of the unknowns with the standards directly on the chromatograms (see above: chromatography). After cooling, 1.25 ml. phosphoric acid are added to

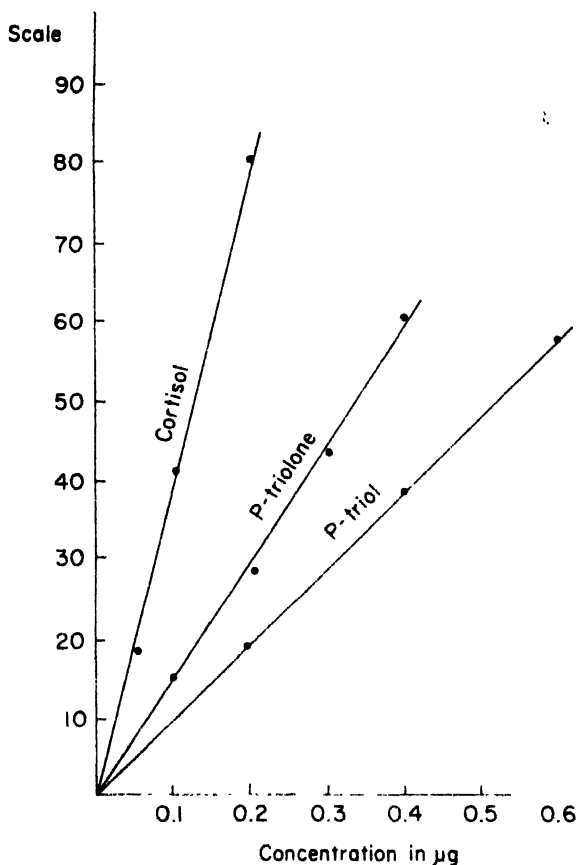


FIG. 2. Fluorescence curves of cortisol, P-triolone (pregnanetriolone and  $3\alpha,17\alpha,20\beta$ -trihydroxy-5 $\beta$ -pregnan-11-one), and P-triol (pregnanetriol). The fluorescence was obtained by heating the respective steroids with 86% phosphoric acid. The recording was made with a Farrand fluorometer.

each tube and the tubes are closed tightly with stoppers lubricated with Dow Corning "silicone lubricant." The tubes are placed in the rack of the water bath which is then immersed in the lighttight boiling water for 15 minutes. The rack is rotated for about 30 seconds at 2, 5, 10, and 14 minutes after starting the reaction. The tubes are rapidly cooled in an ice bath and the contents transferred to cuvettes for fluorometry. With

each set of samples to be estimated an appropriate standard steroid and a "reagent blank" are run. Usually the standard is prepared at three different concentrations ranging between 0.1 to 0.5  $\mu\text{g.}$ , and by direct comparison the concentrations of the unknown are calculated. The readings are made in a Farrand fluorometer using a combination of glass filters with maximum transmission at 425  $m\mu$  in the primary position, and a Farrand interference filter transmitting at 535  $m\mu$  in the secondary position. The intensities of the fluorescence of cortisol, pregnanetriolone, and pregnanetriol differ from each other, but in all cases are proportional over a wide range to the respective concentrations. Both 20 $\alpha$ - and 20 $\beta$ -pregnanetriolones show similar fluorescence (Fig. 2).

**VII. Method II: Quantitative Estimation through the  
Determination of Acetaldehyde ("Acetaldehydogenic" Steroids)  
(Cox, 1959)**

This method is specific for glycols having the structure  $\text{CH}_3 \cdot \text{CHOH} \cdot \text{C}(\text{OH})\text{R}'\text{R}''$ , and among known steroids, for  $\text{C}_{21}$  deoxy compounds possessing hydroxy groups at C-17 and C-20. Thus all steroids listed in Table I may be estimated.

**PROCEDURE**

Sections of the second chromatogram (see Section IV, C) containing the acetaldehydogenic steroids are cut out and transferred into oxidation tubes. Equal volumes of 0.12 *M* periodic acid in 0.4 *N* sulfuric acid and 1% glycine in 0.4 *N* sulfuric acid are freshly mixed and 1 ml. of the mixture pipetted into each oxidation tube. The aeration head of the tube is connected immediately and a trap tube (10-ml. conical centrifuge tube) containing 0.5 ml. 1% sodium bisulfite solution is placed in position to collect the formed acetaldehyde. A slow stream of purified air is passed through the oxidation tube. After 1 hour the aeration is discontinued and 3.5 ml. of concentrated sulfuric acid are added to the bisulfite trap solution. After mixing, the solution is cooled in an ice water bath and then 0.02 ml. of 4% copper sulfate solution and 0.02 ml. of 1.5% 4-hydroxydiphenyl solution in 0.1 *N* NaOH are added. The solution is stirred with a glass rod and kept in the ice bath for 1 hour, with repeated stirring after 30 minutes. Excess 4-hydroxydiphenyl is destroyed by heating the tube in a boiling water bath for 1.5 minutes. The optical density of the solution at 565  $m\mu$  is determined and compared to a calibration curve of a pregnanetriol standard. Several

precautions must be taken in this color reaction to exclude interference (Cox, 1952). As little as 2  $\mu$ g. of any of the above-mentioned steroids may be quantitatively estimated. Results obtained with this method compare well with the fluorometrical estimation.

### VIII. Other Methods

Pregnanetriolone may be indirectly estimated by a determination of 17-ketosteroids formed after oxidative removal of the side chain. Methods using chromic acid (Bongiovanni *et al.*, 1959) or a bismuthate oxidation (Bergstrand *et al.*, 1959) have been described. Because of their limited sensitivity and low specificity the use of these methods is not recommended for analyses of urines containing microgram quantities of pregnanetriolone per 24-hour sample.

### IX. Occurrence of Pregnanetriolone

Estimation of pregnanetriolone was performed on urine of the following cases: (a) normal males and females; (b) pregnancies in the third trimester; (c) hirsutism, otherwise without virilization; (d) hermaphroditism, male pseudohermaphroditism, and female pseudohermaphroditism of constitutional type; (e) adrenal adenoma and adrenal carcinoma; (f) virilizing syndrome due to congenital adrenal hyperplasia; (g) Cushing's syndrome with and without adrenal hyperplasia; (h) virilizing syndrome due to clinically unexplained reasons; (i) Stein-Leventhal syndrome.

### X. Cases Where Pregnanetriolone Was Not Found (Table II)

#### A. NORMAL PERSONS

Out of several hundred analyses performed on the urine of normal persons not a single case was found containing pregnanetriolone (Finkelstein and Goldberg, 1957; Cox and Finkelstein, 1957; Birke *et al.*, 1958; Finkelstein, 1959; Cox, 1960, and personal communication). In most of them, methods I and II were employed which have a sensitivity of 50  $\mu$ g. pregnanetriolone in a 24-hour urine sample. In several instances, method Ia was used by which 2  $\mu$ g./24-hour urine sample could be estimated. Even then no pregnanetriolone was detected.



### B. STIMULATION WITH ACTH

Seven normal persons received intramuscular injections of 100 mg. ACTH-gel daily for 3 days. None of the treated subjects excreted pregnanetriolone before, during, or after medication. The adrenal response was followed up by the parallel estimation of 17-ketosteroids and pregnanetriol and in most cases there was a considerable increase. Pregnanetriolone has also been investigated in the urine of patients with pemphigus vulgaris receiving up to 150 mg. ACTH daily for 5 days as therapy. In no instance was pregnanetriolone detected, although in some of the estimations the sensitivity of the method was 2  $\mu$ g./24 hours.

Results obtained on the urine of subjects who had been stimulated with ACTH suggest that normally the excretion of pregnanetriolone must be below 1  $\mu$ g. in a 24-hour urine sample although stimulation with ACTH produced 17-ketosteroid and pregnanetriol increases of two- to threefold. On assuming that the excretion of pregnanetriolone is influenced in a similar way, 0.7  $\mu$ g. in 24 hours would increase to about 2  $\mu$ g. Such a quantity should be detected by the method employed, but was never found. Proof that, in certain pathological cases, ACTH stimulates the excretion of pregnanetriolone will be given below (see Section XI, A-D).

### C. PREGNANCY

Urine from 200 pregnant women in the third trimester did not contain detectable pregnanetriolone (Finkelstein and Goldberg, 1957).

### D. HIRsutISM

Forty patients with simple hirsutism, but no virilization and with menstrual disturbances in some instances, did not excrete measurable pregnanetriolone (Finkelstein, 1959 and unpublished).

### E. HERMAPHRODITISM AND PSEUDOHERMAPHRODITISM DUE TO EXTRA-ADRENAL FACTORS

Pregnanetriolone was not found in one case of true hermaphroditism (Zachariae, 1955), in two cases of male pseudohermaphroditism (Zondek and Finkelstein, 1954), nor in one case of female pseudohermaphroditism of the constitutional type (Finkelstein, 1959).

## F. ADRENAL ADENOMAS AND CARCINOMA

Especially noteworthy is the absence of pregnanetriolone in the urine of most cases of adrenal neoplasia. Altogether 14 cases of carcinoma and 7 cases of adenomas were studied. These patients had a wide range of clinical manifestations such as virilization, Cushing's syndrome, Conn's syndrome, and symptoms not pertaining to the secretions of the adrenal hormones. In some, in addition to the determinations of pregnanetriolone, the urine was analyzed for pregnanetriol, cortisol, and 17-ketosteroids. The particulars are summarized in Tables III to V. It is of interest to

TABLE II

CASES IN WHICH VIRTUALLY NO PREGNANETRIOLONE WAS EXCRETED

No. of cases	Condition	P-triolone <sup>a</sup> (mg., 24 hours)	P-triol <sup>b</sup> (mg./24 hours)
280	Normal adults	None	0.1-2.0
7	Normal adults after ACTH 3 days; 100 mg. I.M./day	None	1.2-3.6
2	Penphigus vulgaris after ACTH 5 days; 150 mg. I.M./day	None	1.1-3.0
200 (30 individuals and pool of 200)	Pregnancies 3rd trimester	None	0.5-2.5
40	Hirsutism	None	0.5-3.5
14	Adrenal carcinoma	None	0.2-1.3
6	Adrenal adenoma	None	0.8-18.9
1	Adrenal adenoma- "Conn's syndrome"	0.1?	2.0

<sup>a</sup> P-triolone — 3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -trihydroxy-5 $\beta$ -pregnan-11-one.

<sup>b</sup> P-triol — 5 $\beta$ -pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol.

note that many of the above cases excreted a considerably increased amount of pregnanetriol. The level was comparable to that seen in cases of adrenal hyperplasia (see Section XI, A) and thus estimation of pregnanetriol alone is in our opinion of little value in a differentiation between adrenal tumors and adrenal hyperplasia. This is contrary to the observation of Bongiovanni *et al.* (1959) that pregnanetriol is usually not increased in adrenal neoplasia.

The absence of pregnanetriolone in 20 of 21 of these cases suggests that it is excreted relatively rarely by patients with adrenal tumors. The only case where it was found was a patient with adrenal adenoma

TABLE III  
VIRILIZING AND FEMINIZING SYNDROMES DUE TO ADRENAL ADENOMA AND CARCINOMA

Patient	Sex	Age (years)	Clinical observation relating to hormonal effects	Adrenal pathology	P-triolone <sup>a</sup> (mg. 24 hours)	P-triol <sup>a</sup> (mg. 24 hours)	17-Ketosteroids (mg. 24 hours)	Reference
Ch.M.	F	58	Virilization	Adenoma	None	---	16-24	Zondek <i>et al.</i> (1957)
M.L.	F	24	Virilization	Adenoma	None	3.0	45	Cox, unpublished
S-Ta	F	9	Virilization	"Adrenal adenoma" of ovary	<0.05	3.0	---	Cox and Finkelstein (1957)
G.H.	M	16 months	Phallus slightly enlarged	Carcinoma	<0.04	0.2	100-160	Finkelstein and Shoenberger (1959)
L.St.	F	55	Virilization	Carcinoma	<0.1	3.4	40-50	Finkelstein and Shoenberger (1959)
M.W.	F	53	Virilization	Carcinoma	<0.05	6.4	200-300	Cox (1959)
J.M.	M	43	Feminization	Carcinoma	<0.05	2.4	27	Cox (1959)

<sup>a</sup> See Table I.

TABLE IV  
CUSHING'S SYNDROME DUE TO ADRENAL ADENOMA AND CARCINOMA

Patient	Age (years)	Sex	Adrenal pathology	P-triolsone <sup>a</sup> (mg./24 hours)	P-triol <sup>a</sup> (mg./24 hours)	Cortisol	Reference
C.C.	22	F	Adenoma	<0.04	0.66	—	Finkelstein (1959)
V.G.	28	F	Carcinoma	<0.15	5.6	1.9	Finkelstein and Shoenberger (1959)
R.S. <sup>b</sup>	30	M	Carcinoma	<0.11	13.0	0.7	Finkelstein and Shoenberger (1959)
G.T. <sup>b</sup>	33	M	Carcinoma	<0.20	4.5	0.1	Finkelstein and Shoenberger (1959)
J.B.	36	M	Carcinoma	<0.10	4.5	1.04	Finkelstein and Shoenberger (1959)
P.V.	38	F	Carcinoma	<0.13	6.1	0.57	Finkelstein and Shoenberger (1959)
V.F.	48	F	Carcinoma	<0.16	1.00	0.008	Finkelstein and Shoenberger (1959)
G.G.	30	F	Carcinoma	None	4.1	3.3	Fukushima <i>et al.</i> (1960)
L.S.	53	F	Carcinoma	<0.04	—	—	Finkelstein (unpublished)

<sup>a</sup> See Table I.

<sup>b</sup> Cushingoid appearance

TABLE V  
ADRENAL ADENOMA AND CARCINOMA ASSOCIATED WITH "ALDOSTERONISM" (CONN'S SYNDROME),  
AND WITHOUT CLINICAL MANIFESTATIONS PERTAINING TO HORMONAL ACTION

Patient, sex, age (years)	Clinical observation relating to hormonal effects	Adrenal pathology	P-triolone <sup>a</sup> (mg./24 hours)	P-triol <sup>a</sup> (mg./24 hours)	Cortisol (mg./24 hours)	17-Ketosteroids (mg./24 hours)	Reference
Ch.O., F, 30	Conn's syn- drome	Adenoma	0.1?	2.0	—	20	Finkelstein and Shoenberger (unpublished)
J.M., F, 56	No clinical evidence of hormonal effects	Malignant adenoma	<0.04	18.9	—	30-35	Finkelstein (1959)
L.M., F, 35	No clinical evidence of hormonal effects	Malignant adenoma	<0.04	5-9	—	20-30	Finkelstein (1959)
J.H., F, 18	No clinical evidence of hormonal effects	Carcinoma	<0.14	5.8	0.5	3-10	Finkelstein and Shoenberger (1959)
T.R., M, 59	No clinical evidence of hormonal effects	Carcinoma	<0.2	6.7	0.3	80-150	Finkelstein and Shoenberger (1959)

<sup>a</sup> See Table I.

associated with hyperaldosteronism—"Conn's disease" (Table V). The excretion was about 0.1 mg./24 hours and thus too small a quantity for complete identification. Of considerable interest are the steroid excretion studies performed by Fukushima *et al.* (1960) on a patient with Cushing's syndrome due to adrenal carcinoma. This patient apparently did not excrete pregnanetriolone (cf. Table IV, patient G.G.), but small amounts of pregnanetetrol and its 20 $\beta$ -epimer were found. Since there seems to be no principal difference in biosynthetic pathways of both pregnanetriolone and pregnanetetrol (see Section XII), the finding of pregnanetetrol may be regarded as indirect evidence for the possibility of pregnanetriolone production by some adrenal tumors. In fact it seems most probable that some tumors may produce steroids, among them pregnanetriolone, which are not usually found under normal conditions. In this respect special consideration should be given to adrenal tumors originating from adrenal hyperplasia (Hannwi *et al.*, 1957). Thus a classification of the above cases as excreting "no pregnanetriolone" must be subject to further studies, but it seems logical to assume, on the basis of past experience, that most additional cases will show a similar pattern of excretion. In this connection it is necessary to point out that by "no pregnanetriolone" we do not intend to imply the complete absence of pregnanetriolone. The term is merely used to indicate that with present techniques pregnanetriolone was not detected.

## XI. Conditions in Which Pregnanetriolone Is Excreted

### A. CONGENITAL ADRENAL HYPERPLASIA (Table VI)

Urine of 30 cases of congenital adrenal hyperplasia (female pseudo-hermaphrodites or males with macrogenitosomia precox) was analyzed by the present author for pregnanetriolone (Finkelstein, 1959 and unpublished). In all of them pregnanetriolone was detected. The urinary excretion in the majority of cases of untreated adult patients varied between 3 to 12 mg./24 hours. Usually with decreasing age, less has been found than in adult patients, especially before age five. In a few cases of adrenal hyperplasia in newborn infants pregnanetriolone has been found in the urine a few days after birth, and varied in amount between 0.05 to 0.2 mg./24 hours. The presence of pregnanetriolone at such an early age may be of considerable diagnostic importance, since it has been observed that in some cases it was most useful in evaluating the condition (Cox and Finkelstein, 1957; Finkelstein, 1959; Bergstrand *et al.*, 1959). Cox (1959, 1960, and personal communication) found pregnanetriolone in all 9 cases of congenital adrenal hyperplasia. They

TABLE VI

EXCRETION OF PREGNANETRIOLONE, PREGNANETRIOL, AND 17-KETOSTEROIDS BY PATIENTS WITH CONGENITAL VIRILIZING SYNDROME<sup>a</sup>

No. of cases	Age	P-etriolone <sup>b</sup> (mg./24 hours) <sup>c</sup>	P-etriol <sup>b</sup> (mg./24 hours) <sup>c</sup>	17-Ketosteroids (mg./24 hours) <sup>c</sup>	Remarks
9	2 days- 10 months	0.06-0.9	<0.03-1.7	0.5-42	Two cases of macrogenitosomia precox, one of them with hypertension; 1 case of "female pseudohermaphroditism" with hypertension; and 1 with "salt-losing" syndrome. All others—female pseudohermaphrodites with no other defects
4	1-2 years	0.2-12	0.03-23	3-9	One case—macrogenitosomia precox with salt losing. All others female pseudohermaphroditism
7	3-6 years	0.2-5	0.7-6	3-40	One case—macrogenitosomia precox; all others female pseudohermaphroditism
3	8-15 years	1-20	4-60	5-50	One case—macrogenitosomia precox; two—female pseudohermaphroditism
16	Adults	1-18	3-60	30-60	All cases—female pseudohermaphroditism

<sup>a</sup> The data include values kindly communicated by Dr. R. I. Cox.<sup>b</sup> See Table I.<sup>c</sup> The validity of the lower limits, especially in the older patients is not certain in view of the probability that some of them were unaware of having been treated previously with cortisone.

included infants aged 1 week to 2 years, children 3 to 15 years, and adults of both sexes. The limits of excretion ranged between 0.07 to 20 mg./24 hours, the lowest amount being excreted by an infant aged 1 week. Bergstrand *et al.* (1959) described excretion of pregnanetriolone in 3 infants and 3 children. No exact quantitative data were reported, but it was noted that the ratio of pregnanetriolone to pregnanetriol excreted was high in the infants and lower in the older children. This confirms the earlier observation of Cox and Finkelstein (1957), and similar results were found by Bongiovanni *et al.* (1959) in their study of 11 patients, aged 2 weeks to 23 years, with congenital adrenal hyperplasia. Further studies on the excretion of pregnanetriolone in congenital adrenal hyperplasia were reported by Fukushima and Gallagher (1957) and by Bush *et al.* (1957).

In addition to pregnanetriolone the excretion of pregnanetrol in the congenital adrenogenital syndrome has been frequently noted (Fukushima and Gallagher, 1957; Cox, 1960). The metabolic relation of these two steroids will be discussed below.

Cortisone or its analogs depresses the excretion of pregnanetriolone very efficiently. The decrease is already evident, in some cases, after a few days' therapy, but in others it may take longer to be significantly decreased. In no case was excretion of pregnanetriolone completely suppressed, though in some cases treatment with cortisone was quite intensive. The most intensively suppressed adult patients still excreted 0.1-0.5 mg. pregnanetriolone in a 24-hour urine sample. In some of them the decrease in the pregnanetriolone excretion was well correlated with the decrease in 17-ketosteroids and pregnanetriol, but in a few pregnanetriolone decreased first, and in still others a daily dose of cortisone was sufficient to decrease the 17-ketosteroids or pregnanetriol without appreciably changing the level of pregnanetriolone. In one case, which was closely followed up, of an adult female pseudohermaphrodite due to congenital adrenal hyperplasia the menstrual period did not occur until the initial 50 mg. per day dose of cortisone was increased to 75 mg., which was sufficient to depress the excretion of pregnanetriolone to about 0.1 mg./24-hour urine sample. During medication with 50 mg. cortisone, pregnanetriolone was excreted at the rate of 1 to 2 mg./24 hours, and the 17-ketosteroids were in the normal range. The latter did not change when the dose of cortisone was increased.

Though the above cases of congenital adrenal hyperplasia, who excreted pregnanetriolone, comprised a wide spectrum of variants of the syndrome such as simple virilization, hypertension, and salt-losing syndrome, it would be unwise to conclude that pregnanetriolone is excreted in all forms of congenital adrenal hyperplasia. In fact the presence of pregnanetriolone is attributed to an impaired hydroxylation at C-21 of the steroid molecule in the course of the biosynthesis of cortisol



(Finkelstein, 1959). On the other hand it has been established that in certain cases of the hypertensive form of the syndrome the hydroxylation at the C-11 may be blocked. If this block is absolute, pregnanetriolone could not be formed. One such case was described in 1955 by Eberlein and Bongiovanni, but analysis for pregnanetriolone was not performed. These authors (Bongiovanni *et al.*, 1959) later found an increased pregnanetriolone in one case of hypertension associated with congenital adrenal hyperplasia, but no conclusion could be reached because the infrared spectrum (performed on crude material) was different from pregnanetriolone. Nevertheless a possibility of congenital adrenal hyperplasia with or without low excretion of pregnanetriolone must be considered. One of the cases with hypertension seen by us had a considerably lower pregnanetriolone excretion (0.15 mg./24 hours) than usually encountered in the simple form of congenital adrenal hyperplasia. Also Cox (1960) noted a comparatively low excretion of pregnanetriolone in one hypertensive patient with congenital adrenal hyperplasia. These patients are similar to the one with hypertension associated with congenital adrenal hyperplasia, reported by Dyrenfurth *et al.* (1958) where small amounts of steroids with oxygen at C-11 were found in the urine. Such cases would belong to a category where a partial deficiency of the 11 $\beta$ -hydroxylase is shown, and would be analogous to the more common deficiency in 21-hydroxylase, which as far as known is never complete. On the other hand very few cases of congenital adrenal hyperplasia showing a complete enzymatic impairment in the C-11 hydroxylation have been described (Green *et al.*, 1960), and may be regarded as exceptional. Hence, in the majority of congenital adrenal hyperplastic patients, pregnanetriolone may be expected to appear in the urine.\*

#### B. CUSHING'S SYNDROME DUE TO BILATERAL ADRENAL HYPERPLASIA (Table VII)

Pregnanetriolone has been determined in 7 cases of Cushing's syndrome due to adrenal hyperplasia, 4 verified by histological examination and 3 seen with enlarged adrenals; it was present in all of them. The excretion varied between 0.05 to 0.5 mg. in a 24-hour urine sample. This is appreciably less than is found in most cases of the virilizing syndrome due to congenital adrenal hyperplasia. The excretion of pregnanetriol in Cushing's syndrome was either within the normal limits or, rarely, slightly elevated. Administration of ACTH to two Cushing's syndrome patients increased the excretion of pregnanetriolone about

\* Note added in proof. Recently we encountered one case of congenital adrenal hyperplasia associated with hypertension which did not excrete measurable pregnanetriolone. On the other hand the urine contained 25 mg./liter of 5 $\beta$ -pregnane-3 $\alpha$ , 17 $\alpha$ , 21-triol-20-one (Tetrahydro Reichstein's compound 8). This case confirms the findings of Eberlein and Bongiovanni (1955) that in this type of congenital adrenal hyperplasia the hydroxylation at C-11 position of certain precursors of cortisol may be practically completely inhibited.

TABLE VII

CUSHING'S SYNDROME ASSOCIATED WITH ADRENAL HYPERPLASIA OR  
WITH UNPROVEN ADRENAL PATHOLOGY

Patient	Sex	Age (years)	Adrenal pathology	P-triolone <sup>a</sup> (mg./24 hours)	P-triol <sup>a</sup> (mg./24 hours)
Ah.A	M	13	Hyperplasia	0.09	0.5-1
G.H.	F	30	Hyperplasia	0.06-0.08	1-3.2
B.L.	F	36	Hyperplasia	0.5	3.5
M.S.	F	38	Hyperplasia	0.06	0.8
J.M.	F	23	Hyperplasia <sup>b</sup>	0.05	1.5
J.G.	M	26	Hyperplasia <sup>b</sup>	0.10	—
E.P.	F	45	Hyperplasia <sup>b</sup>	0.05	0.93
N.G.	M	32	Unproven	0.26-0.3	1-2.8
E.M.	F	35	Unproven	0.11	0.22
E.Sh	F	18	Unproven	0.08	0.45
R.Sh	F	34	Unproven	0.26	0.80
E.V.	F	52	Unproven	0.08	0.70
V.L.	F	19	Unproven	0.075	0.53
Seven cases	F	14-57	Unproven	None	0.4-1.5

<sup>a</sup> See Table I.

<sup>b</sup> Indicated by perirenal insufflation.

threefold. This increase was not accompanied by an increase of pregnanetriol. Curiously, in one case pregnanetriol markedly decreased during the increase in pregnanetriolone excretion (Finkelstein, 1959).

Of some interest was the remarkable correlation between the clinical condition and the excretion of pregnanetriolone observed in one patient with bilateral adrenal hyperplasia. The initial excretion during the pre-treatment period was 0.09 mg./24 hours. After a bilateral subtotal adrenalectomy the level of pregnanetriolone decreased to a level not detectable by our method. Control estimations carried out during the following 2 years did not detect any pregnanetriolone. At the end of these 2 years, when the clinical condition rapidly deteriorated, 0.07 mg./24 hours of pregnanetriolone could be detected. The patient was operated on again and a total adrenalectomy was performed. No pregnanetriolone was found postoperatively (Ehrenfeld and Finkelstein, unpublished).

#### C. CUSHING'S SYNDROME WITH NO EVIDENCE OF ADRENAL HYPERPLASIA (Table VII)

In 6 of the 13 patients with Cushing's syndrome without evidence of adrenal hyperplasia, pregnanetriolone was excreted in the urine in the

range 0.04–0.30 mg./24 hours. In contrast, the other 7 patients with similar clinical symptoms did not excrete detectable pregnanetriolone. Stimulation of the adrenals of one of these patients with ACTH in a way similar to the previous cases did not result in any occurrence of urinary pregnanetriolone. This observation shows that “hypercorticism” per se, as it undoubtedly exists in the Cushing’s syndrome, does not necessarily result in the excretion of pregnanetriolone. Even further stimulation of the adrenals which usually are hypersensitive to ACTH (Nugent, 1960) may not influence the excretion of pregnanetriolone.

Thus the presence of pregnanetriolone in certain patients with Cushing’s syndrome and its absence in others (the discussion here is limited only to the syndrome *not due* to neoplasia), suggests that the syndrome may comprise several biochemical variants with similar clinical manifestations, but of different etiology.

It seems reasonable to assume that when pregnanetriolone is present, the primary lesion lies within the adrenals, and is due to an enzymatic defect which is imperative for the formation of the compound. Quite probably this may be an inborn deficiency, which becomes evident in postnatal life, and especially postpubertally. A congenital enzymatic lesion has been similarly postulated in the case of postnatal (pre- or postpubertal) virilizing syndrome due to adrenal hyperplasia (Wilkins, 1957; Greenblatt, 1958; Brooks *et al.*, 1960). It is of interest to note that the adrenals of these patients are not always enlarged, although they continue to be classified, somewhat strangely, as “adrenal hyperplasia” (Brooks *et al.*, 1960).

In contrast to the above cases of Cushing’s syndrome in which an enzymatic deficiency is suspected, some of the cases not excreting pregnanetriolone, when stimulated with ACTH, may be regarded as simple “hypercorticism” and are probably due to a small excess of ACTH. Such an explanation of Cushing’s syndrome associated with adrenal hyperplasia has been recently considered by Nugent *et al.* (1960).

#### D. POSTNATAL VIRILIZATION SYNDROME AND STEIN-LEVENTHAL SYNDROME (Table VIII)

The etiology of the postnatal virilization syndrome, other than that conditioned by adrenal or gonadal tumors, remains uncertain.

Some authors believe that the postnatal adrenal virilism may be due to “acquired hyperplasia” (Dorfman and Shipley, 1956), and others prefer to regard it as a latent congenital lesion (Greenblatt, 1958).

Relatively few adrenals of these cases have been explored and they have not always been found to be hyperplastic (Brooks *et al.*, 1960).

TABLE VIII

POSTNATAL VIRILIZING SYNDROME WITH UNPROVEN ADRENAL PATHOLOGY

Patient	Sex	Age (years)	P-triolone <sup>a</sup> (mg./24 hours)	P-triol <sup>a</sup> (mg./24 hours)	17-Ketosteroids (mg./24 hours)
L.G.	F	51	0.08	0.86	5.4
Ca.K.	F	20	0.25	3.2	34.9
A.R.	F	18	0.13-0.2	0.7-1	12.2
J.S.	F	35	0.4-1.0	3.5-5.0	18-22
N.P.	F	18	0.1-0.26	1-1.2	12
Sh.F.	F	18	0.8 <sup>b</sup>	4.5 <sup>b</sup>	10 <sup>b</sup>
R.N.	F	19	<0.04	9.0	14.8
Ch.F.	F	28	<0.04	4.3	16.8
Eleven cases	F	8-28	None	0.2-2.5	5-15

<sup>a</sup> See Table I.

<sup>b</sup> After intramuscular administration of 100 mg. ACTH-gel, daily for 3 days. Before ACTH was given no detectable pregnanetriolone was excreted; the mean level of pregnanetriol was 2.6 mg./24 hours and 17-ketosteroids, 10 mg./24 hours.

The excretion of the 17-ketosteroids and pregnanetriol is variable and may be increased in some cases but normal in others.

Pregnanetriolone has been estimated by us in 19 cases of postpubertal virilizing syndrome. Most of them did not excrete detectable pregnanetriolone. In the 5 positive cases the excretion was in the range of 0.1 to 1.0 mg. of pregnanetriolone per 24-hour urine sample. Only in 4 cases was pregnanetriol significantly increased and, in 2 of them, high pregnanetriol was not associated with a corresponding increase in pregnanetriolone. Additional cases of postpubertal virilizing syndrome excreting pregnanetriolone were presented by Jayle *et al.* (1958), and Brooks *et al.* (1960).

Stimulation with ACTH, in one of our cases, with prior absence of pregnanetriolone, resulted in its increase to measurable levels (Sh.F., Table VIII). In the others, where pregnanetriolone was initially present in small amounts it was further increased after administration of ACTH. Of special interest one of them (N.P., Table VIII) responded to 100 mg. of ACTH-gel, given intramuscularly by an increase from the initial level of 0.2 mg. of pregnanetriolone in 24 hours to 5.3 mg. in 24 hours. Pregnanetriol rose from 1 to 27 mg. in 24 hours, and 17-ketosteroids from 12 to 35 mg. in 24 hours. The twenty-five-fold increase of pregnanetriol and pregnanetriolone strongly implies an inhibition in the C-21 hydroxylation of one of the precursors of cortisol. The fact that the 17-ketosteroids increased by only a factor of 3 during the same period may be taken as evidence against the concept of a general oversensitivity

of the adrenals. Brooks *et al.* (1960) obtained similar but less dramatic results by stimulating with ACTH 3 patients who had postpubertal virilizing syndrome. The increase of pregnanetriol was by a factor of 2, 5, and 6, respectively. The 17-ketosteroids rose in 2 cases to about twice the initial level, and remained unchanged in the third. No data were presented on pregnanetriolone after the stimulation, though in two cases pregnanetriolone was initially present in concentrations of 0.9 and 1.0 mg. per 24-hour sample. The main difference between the cases of Brooks *et al.*, and our series is the high "resting" values of pregnanetriol noted by the first-mentioned authors which distinguished the patients from normal. Our results show the occurrence of a "latent" adrenogenital syndrome with normal pregnanetriol and 17-ketosteroids, but with an abnormal excretion of pregnanetriolone. However, by exposing the patient to an excess of ACTH a condition was created which, as far as the urinary steroid excretion is concerned, was indistinguishable from the congenital form of the virilizing syndrome.

Pregnanetriolone has occasionally been found in the urine of patients with Stein-Leventhal syndrome (Cox, personal communication;<sup>1</sup> Finkelstein and Shoenberger, unpublished results). No immediate explanation can be offered why some patients do and others do not excrete this steroid. The possibility that an abnormal function of the adrenal cortex in these patients (Greenblatt, 1958) causes excretion of pregnanetriolone must be considered. In contrast, one of our patients with Stein-Leventhal syndrome who excreted pregnanetriolone prior to ovarian resection ceased gradually to excrete the compound following the operation. No plausible explanation can be offered at this stage.

## XII. Biosynthesis of Pregnanetriolone

The most probable biosynthetic precursor of pregnanetriolone is either 21-deoxycortisone or 21-deoxycortisol. This has been clearly shown by several investigators (Jailer *et al.*, 1955; Rossetti *et al.*, 1957; Fukushima *et al.*, 1958, 1959; Ungar *et al.*, 1960), who isolated pregnanetriolone and pregnanetetrol in urine after administration of 21-deoxycortisol and 3 $\alpha$ ,17 $\alpha$ -dihydroxy-5 $\beta$ -pregnane-11,20-dione, respectively, to persons with normal endocrine functions and to patients with congenital adrenal hyperplasia. Thus it is beyond doubt that 21-deoxycortisol, if present in the human body, is normally converted to preg-

<sup>1</sup> After submission of this article for publication R. P. Shearman, R. I. Cox, and A. Gannon reported that in sixteen consecutive cases of Stein-Leventhal syndrome pregnanetriolone was found in the urine [*Lancet*, i 260, 1961].

nanetriolone. On the other hand Finkelstein and Goldberg (1957) and Fukushima and Gallagher (1958) demonstrated that neither cortisone nor cortisol may be regarded as precursors of pregnanetriolone. This verifies the hypothesis of Dorfman (1955) that the main defect in the most common type of congenital adrenal hyperplasia (and in some cases also in other types of adrenogenital syndrome, see above) is due to some insufficiency in the enzyme system involved in the hydroxylation at the C-21 position. This hydroxylation must normally take place in the biosynthetic reactions leading to the formation of cortisol before the hydroxylation at C-11 (Hayano *et al.*, 1956). Had the C-11 hydroxylation been the first in the sequence, the formation of 21-deoxycortisol would lead to excretion of pregnanetriolone. But normally, until now, no pregnanetriolone could be found in the urine. Even an energetic stimulation of the adrenal cortex with ACTH, causing a significant overproduction of other steroids, did not bring about excretion of pregnanetriolone (Finkelstein, 1959). Neither is the compound excreted in cases of adrenal tumors showing elevated secretion of steroids. On the other hand in some of these cases pregnanetriol was significantly increased. The major precursor of pregnanetriol is most probably 17 $\alpha$ -hydroxyprogesterone (Fotherby and Love, 1960). The increased excretion of pregnanetriol may be regarded as a result of the overflow of 17 $\alpha$ -hydroxyprogesterone, which accumulates either under the influence of the exogenous ACTH stimulation or of the neoplastic tissue. Since the 21-hydroxylase cannot cope with such an excess of 17 $\alpha$ -hydroxyprogesterone, a part of it is reduced to pregnanetriol. A possible explanation as to why this extra 17 $\alpha$ -hydroxyprogesterone has not been exposed to the action of 11 $\beta$ -hydroxylase to form 21-deoxycortisol is outlined below.

The situation is different in virilizing syndrome due to congenital adrenal hyperplasia, and excreting pregnanetriolone. Here the normal biosynthesis of cortisol is obstructed by the deficient C<sub>21</sub> hydroxylase (Dorfman, 1955), which is essential for conversion of 17 $\alpha$ -hydroxyprogesterone to the intermediate 11-deoxycortisol. In contrast to events under normal conditions, a part of the 17-hydroxyprogesterone becomes hydroxylated at the C-11 position and transformed into 21-deoxycortisol. The last-mentioned compound is poorly converted (if at all) into cortisol and largely excreted as pregnanetriolone (Fukushima *et al.*, 1959). Thus two major metabolites are excreted in the urine: pregnanetriol originating from the accumulated 17 $\alpha$ -hydroxyprogesterone, and pregnanetriolone from the abnormally produced 21-deoxycortisol. Accordingly, the last-mentioned compound should be regarded as the "abnormal hormone" in congenital adrenal hyperplasia, with a deficiency in the C-21 hydroxylating system.

In cases of virilizing syndrome arising postpubertally, and excreting pregnanetriolone, a similar enzymatic defect may be considered, except that the relative insufficiency of the 21-hydroxylase is less pronounced. Thus the production of cortisol is impaired to a lesser degree, with a subsequently more efficient inhibition of ACTH secretion than in the congenital variant of the syndrome. It follows, that in the postpubertal virilizing syndrome the adrenals are generally exposed to less stimulation by ACTH than in the congenital form. If so, the rate of biosynthesis of the  $C_{21}$  steroids, among them  $17\alpha$ -hydroxyprogesterone, should be lower than in congenital adrenal hyperplasia. Concurrently less pregnanetriol should be excreted. The experimental results are in agreement with the above postulate. However, the occurrence of pregnanetriolone in postpubertal virilism deserves special consideration. Since a considerable increase of  $17\alpha$ -hydroxyprogesterone cannot be expected in this syndrome, and, on the basis of urinary pregnanetriol excretion, it may even not be increased at all, the formation of pregnanetriolone cannot be due to excess  $17\alpha$ -hydroxyprogesterone. On the other hand, if the assumption is made that in the adrenogenital syndromes excreting pregnanetriolone, an abnormal  $11\beta$ -hydroxylase specific for  $C_{21}$  deoxysteroids is functioning, the occurrence of pregnanetriolone can easily be understood. In other words, two different and independent enzymatic lesions are operative in the various adrenogenital syndromes (with the excretion of pregnanetriolone). The first would be the relative deficiency in the 21-hydroxylase, and the second, the availability of an  $11\beta$ -hydroxylase specific for  $C_{21}$  deoxysteroids.

In the most common congenital virilizing adrenal hyperplasia the deficiency in the  $C_{21}$  hydroxylase leads to an excess of  $17\alpha$ -hydroxyprogesterone, which is in part excreted as pregnanetriol and in part converted by the unusual  $11\beta$ -hydroxylase to pregnanetriolone and pregnanetetrol (via 21-deoxycortisol). These compounds are excreted at a greatly increased level.

In the postpubertal virilizing syndrome, the relative insufficiency of 21-hydroxylase is much less, but, due to the presence of the abnormal  $11\beta$ -hydroxylase acting upon  $17\alpha$ -hydroxyprogesterone, a part of the latter would be converted to 21-deoxycortisol and excreted as pregnanetriolone. Since no serious accumulation of  $17\alpha$ -hydroxyprogesterone occurs, normal or moderately increased amounts of pregnanetriol are excreted.

In Cushing's syndrome, with pregnanetriolone excretion, some of the  $17\alpha$ -hydroxyprogesterone is exposed to the abnormal  $11\beta$ -hydroxylase and eventually transformed into pregnanetriolone. No deficiency has

been observed in the C-21 hydroxylating system, and in most cases the excretion of pregnanetriol remains within normal limits.

Normal persons, receiving ACTH, excrete more pregnanetriol. No pregnanetriolone is formed, because the normal sequence of events leading to the biosynthesis of cortisol by way of  $17\alpha$ -hydroxyprogesterone  $\rightarrow$  11-deoxycortisol is maintained.

To designate this virtually qualitative functional difference in the adrenogenital syndromes, excreting pregnanetriolone, the term *adrenal heterofunction* has been suggested (Finkelstein, 1959). This term may be especially useful to apply to those adrenogenital syndromes, frequently referred to as "adrenal hyperplasia," in which in fact no hyperplasia exists.

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## Chapter 6

# Adrenocorticosteroids

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I. Introduction	199
II. Requirements for a Good Chemical Assay	201
A. General	201
B. Collection and Storage of Samples	203
C. Extraction of Adrenocorticosteroids	204
III. Methods of Assay	215
A. General	215
B. Chemical Methods	218
C. Fluorometric Methods	247
D. Radioisotopic Methods	254
E. Other Methods	256
References	257

### I. Introduction

The past fifteen years have been marked by significant successes of many investigators in isolating and elucidating the structure of adrenocorticosteroids excreted in the urine under normal and pathological conditions. Concomitantly, chemical assay methods were developed which eliminated the necessity of utilizing tedious and difficult classic isolation procedures for quantitation of the steroids. These newer methods are relatively simple and practical; this is due to the fact that many of the adrenocorticosteroids possess functional groups on the steroid nucleus which react readily with easily available and inexpensive chemical reagents. The products of the reaction, which are for the most part colored or fluorogenic, can then be measured with suitable instruments of the spectrophotometer type. Because of the high degree of sensitivity of the chemical assay methods, steroid quantitation can now be carried out with a smaller fraction of the total biological sample than would have been necessary had the classic method of isolation been required.

Recent advances in instrument design have made it possible to increase further the sensitivity of the chemical assay methods; this thus permits the analyses of microamounts of adrenal steroids in biological material. Mechanical attachments designed for the Beckman DU spectrophotometer or a similar instrument, microcells which permit the measurement of color intensities in 1.0 ml. or less of solution, the availability of refined

spectrophotofluorometers as well as infrared spectrophotometers are only a few of the tools available to make steroid quantitation feasible, simple, accurate, and extremely sensitive.

Before the methods and instruments can be used with confidence, it is absolutely necessary for the analyst to understand some of the requirements which will make the chemical assay reliable. One of these, which will be discussed fully later, is the necessity for having several purification methods which can be used to eliminate nonsteroidal material from crude organic solvent-soluble extracts of biological material. By reacting with many of the reagents used in a particular chemical assay these materials interfere with color development or often give rise to colored products whose absorption maxima correspond to those given by the steroids. Consequently, the sensitivity and specificity of a particular method may be greatly decreased.

As a wider spectrum of steroids is isolated from various biological sources, improvement of the existing chemical methods or development of new techniques may be required so that reliable measurements can be done easily either for clinical work or research. This may also necessitate changes or re-evaluations of the existing indices which have been applied to the interpretation of adrenocortical function in normal or pathological states. In a large measure this will depend on the quantity of the steroid(s) found in relation to the metabolic state of the individual.

Exact isolations of the pregnantetrols from urine (Finkelstein *et al.*, 1953; Cox and Finkelstein, 1957; Cox, 1959; Finkelstein, 1959) are a case in point. Looked upon at first as perhaps oddities in terms of urinary steroid metabolites, they have been shown recently to be of value in aiding in the diagnosis of a particular type of adrenal hyperplasia to which Finkelstein (1959) has ascribed the term "adrenocortical heterofunction." The appearance of substantial amounts of cortolone ( $3\alpha,17\alpha,20\alpha,21$ -tetrahydroxy- $5\beta$ -pregnan-11-one) and  $\beta$ -cortolone ( $3\alpha,17\alpha,20\beta,21$ -tetrahydroxy- $5\beta$ -pregnan-11-one) as well as the respective cortol (position 11 hydroxylated) epimers in human urine (Fukushima *et al.*, 1955; Bush and Willoughby, 1957; Romanoff *et al.*, 1959) and their parallel relationship to the urinary excretion of tetrahydrocortisol, allotetrahydrocortisol, and tetrahydrocortisone (Romanoff *et al.*, 1959) indicates that these substances also may be used as indices of adrenocortical function.

In this chapter the author will present some of the basic requirements for reliable chemical assays that may be used for routine analyses. To this end one must necessarily deal with certain fundamental aspects. This chapter has been written principally from the practical point of view for those who wish to use chemical analyses for the determination of adrenocorticosteroids. No special mention will be made concerning the

chemical assay of aldosterone. This subject is outlined competently in Chapter 7 of this book.

Although Section III deals with both old and newer methods, it does not do so as an advanced treatise on the subject. Specialists in the field of chemical analyses probably have supplementary data which cannot appear here as a result of the limitations imposed on the present author by time. Finally, the references quoted were chosen to represent a cross section of significant findings. A representative choice has been necessary for the sake of convenience as it is impossible to quote all pertinent work. For a more extensive bibliography the reader is referred to excellent monographs and reviews by Callow (1950), Lieberman and Teich (1953), Engel (1954), Zaffaroni (1953), Dorfman (1957), Roberts and Szego (1955), Zimmermann (1955), and Saffran and Saffran (1959).

## II. Requirements for a Good Chemical Assay

### A. GENERAL

Although different chemical assay methods may be used for the estimation of the same, or closely related, corticosteroids which are described as formaldehydogenic, reducing, 17-ketogenic, etc., these, regardless of their simplicity or complexity, must satisfy the requirements of specificity, reproducibility, and sensitivity. Of these, the first is the most difficult because the reagents used must react with certain specific chemical groupings on the steroid molecule. Specificity, in a large measure, will depend upon the methods used for extraction and the degree of purification of the adrenocorticosteroids achieved. In addition, to meet the criterion of specificity the results obtained with a given chemical assay method must be consistent with the medical facts. For example, in primary adrenal insufficiency, Addison's disease, or hypopituitarism one would expect the levels of adrenal steroids to be very low and conversely, to be very high in congenital hyperplasia and certain manifestations of Cushing's syndrome.

The second requirement for a chemical assay is reproducibility. This means that a method should yield relatively constant values in replicate determinations of the same sample analyzed on the same or different days. In addition, when used by several groups of workers the results should be in agreement. In this type of experiment, known substances are carried through all the manipulations necessary for obtaining the material one wants to measure either in tissue or biological fluids. This sometimes proves difficult because of the unavailability of the steroid to be determined. For example, at present steroid glucosiduronidates are difficult to

obtain and therefore little work has been carried out to check the accuracy of methods which depend at one stage on the extraction of adrenocorticosteroid conjugates (Reddy *et al.*, 1952; Reddy, 1954). As a rule, adrenal steroids are extracted, after acid or enzyme hydrolyses, in the free form. Since most of these substances are commercially available, the considerations discussed above do not present a serious problem.

The investigator seldom finds it difficult to meet the criteria for greater precision and sensitivity with pure steroids when using a colorimetric assay. This requires that the optical density reading, for the steroids only, should be "within the range which is consistent with good accuracy. In most instances this is between 0.2 and 0.7" (Archibald, 1950, 1953). The interfering substances in extracts of biological material, however, make it almost impossible to meet this criterion due to their reaction with some or all of the reagents used in the chemical assay. In many instances the color produced is so intense (blank color), relative to that given by the steroid alone, that interpretation of the values is difficult. In such cases the investigator has no recourse but to increase the specificity of his method or to attempt further resolution and purification of his extract to eliminate nonspecific material.

Regardless of whether the methods are to be used as research tools or in routine clinical assay work too much emphasis cannot be placed on the above criteria. Other points to be considered are rapidity, simplicity, and cost. These, however, do not affect the primary requirements of a good chemical assay but are dependent on a particular set of circumstances.

Once the requirements for a good assay have been met, consideration must be given to the conditions under which the biological samples were collected. Diurnal variation in adrenal steroid secretion over a 24-hour period has been well established (Pincus *et al.*, 1948; Romanoff *et al.*, 1949, 1957; Jorgensen, 1957). Therefore, short-term urine collections cannot reflect daily secretion patterns since the values will vary with the time of collection. This also applies to blood and tissue studies (Bliss *et al.*, 1953; Doe *et al.*, 1954; Gemzell, 1955; Migeon *et al.*, 1956a; Reddy *et al.*, 1956; Halberg *et al.*, 1958, 1959).

One of the apparent advantages of assaying blood corticosteroids is the possibility of differentiating between administered substances which can cause a protracted, a momentary, or a rapid rise in plasma corticosteroids as a result of an activation of the adrenal glands. Conditions which cause momentary rises in blood adrenocorticosteroids will not be detected by analyzing the urinary corticosteroids of a pooled 24-hour sample of urine. In general, however, good agreement exists between blood and urinary corticosteroid levels (Eik-Nes *et al.*, 1954a, 1955;

Sweat, 1955), such that the former can be relied upon as an index of cortical activity.

It should be understood that these measurements reflect an equilibrium between production (formation or release from the adrenals or other extra-adrenal steroid-producing tissue) and removal (storage, excretion, or breakdown) of adrenal steroids. In the normal individual (without diseases affecting the adrenohypophyseal axis) factors which bring about a change in this equilibrium will be reflected as a rule in either an elevation or a decrease of the steroid level in blood, urine, or tissue. However, caution must be exercised in interpreting changes in corticosteroid secretion since it is possible for factors other than those concerning glandular secretion to influence the level of blood, urine, and presumably tissue steroids.

It is well known that (1) the liver is implicated in the metabolism of adrenocorticosteroids. Complete or partial hepatectomy (Nelson, 1951, Del Greco *et al.*, 1953), liver diseases, cirrhosis, and liver failure (Brown *et al.*, 1954; Hellman *et al.*, 1954; Conn *et al.*, 1954; Birke and Plantin, 1955; Klein *et al.*, 1955; Samuels *et al.*, 1957) prevent the rapid disappearance of administered adrenal steroids from the blood. This is reflected in an increase in free but a decrease in blood steroid conjugates and in the excretion of corticosteroids in urine. (2) Kidney damage significantly affects the levels of adrenal steroid conjugates circulating in blood. As a result the accumulation of the latter significantly affects the rate of conjugation occurring in the liver (Englert *et al.*, 1957). (3) In advanced pregnancy, there is an increased blood level of free corticosteroids attributed to the increased "life" of the free steroid before its conjugation (Migeon *et al.*, 1957). These are three of the more important considerations which reveal that differences in adrenal function may be masked because of compensatory mechanisms which are involved in the metabolism and excretion of steroids. Although these considerations are not directly concerned with the chemical assays per se they should be kept in mind when interpreting the data.

The reader has now been made aware of some of the general pitfalls which are encountered in chemical assay methods and their interpretations. These are of such a nature as to confound the novice who, entering this field of investigation without forewarning, would tend to interpret readings obtained on his densitometer as absolute truths.

## B. COLLECTION AND STORAGE OF SAMPLES

Biological samples, which are not stored in the frozen state, must be preserved against possible bacterial growth to prevent destruction of the

steroids to be assayed. For example, steroid conjugates in urine can undergo hydrolysis by bacterial enzymes such as to alter the ratio of free to conjugated steroids. An acetic acid solution of thymol (1%) is a satisfactory bacteriostatic agent but the presence of acetic acid is undesirable. Because of its extreme solubility in most organic solvents used in extraction procedures it interferes with some of the chemical assays.

Birke *et al.* (1958) have shown that storing urine samples under toluene at room temperature is adequate when determining urinary 17-ketogenic steroids; other methods are also used (Tompsett and Smith, 1955).

As in the case of urine storage, problems are also encountered with storage of blood and tissue. Rapid entry of adrenal steroids into the blood cells of a freshly drawn sample of blood (Migeon *et al.*, 1957) makes the immediate centrifugation of the blood necessary. The plasma may then be stored in the frozen state and thawed prior to its analysis. Tissues may be stored in the frozen state prior to homogenization and extraction with solvent. Alternatively, the tissue may be homogenized immediately with ethanol and the homogenates stored in the cold at a final concentration of 13% ethanol (Silber *et al.*, 1958; Péron and Dorfman, 1958).

The importance of collecting 24-hour urine samples has been stressed above because of diurnal variation. To ensure completeness of urine collection, creatinine determinations should be carried out (Peters and Van Slyke, 1932).

### C. EXTRACTION OF ADRENOCORTICOSTEROIDS

#### 1. *Hydrolysis of Steroid Conjugates*

With the exception of the guinea pig and cat (Burstein *et al.*, 1955; Borrell, 1958; Péron and Dorfman, 1958) the greater proportion of adrenocorticosteroids or their metabolites excreted in urine or circulating in blood are present as water-soluble glucosiduronidate complexes. The latter are insoluble in most organic solvents. For this reason, methods of hydrolyses have been devised which will cleave the complex and liberate the steroids in a solvent-soluble form.

*a. Acid Hydrolysis.* Strong mineral acids have proved invaluable for hydrolyzing 17-ketosteroid conjugates causing little or no destruction of this class (however, see Engel, 1954) of steroid. On the other hand, the adrenal steroids, free or conjugated, are extremely labile in the presence of mineral acids. Destruction of adrenocorticosteroids by acid has been clearly demonstrated (Talbot and Eitingon, 1944; Heard *et al.*, 1946; Lieberman and Dobriner, 1948; Cohen, 1951; Cox and Marrian, 1951; Lieberman *et al.*, 1952). Fukushima and Gallagher (1957a) showed that 17-deoxy compounds arise from 17 $\alpha$ -hydroxy adrenocorticosteroids as



artifacts of acid hydrolysis but not after enzymatic hydrolysis. Tompsett (1953) had taken advantage of this fact to suggest a procedure wherein acid hydrolysis at an elevated temperature permits the determination of 17-deoxy formaldehydogenic steroids specifically. These findings, however, were not substantiated by Marrian and Atherden (1953).

*b. Enzyme Hydrolysis.* Because of the extreme lability of the adrenocorticosteroids in the presence of mineral acids a very mild hydrolytic procedure is necessary, namely, enzyme hydrolysis. This can be accomplished by the enzyme  $\beta$ -glucuronidase which has been isolated from various animal tissues and from bacteria (Talbot *et al.*, 1943; Mason and Kepler, 1945; Buehler *et al.*, 1949; Cohen, 1951; Katzman *et al.*, 1952; Fukushima *et al.*, 1953). This enzyme is available commercially as splenic and bacterial  $\beta$ -glucuronidase, and these preparations hydrolyze the glucosidic bond between glucosiduronic acid and the steroid moiety (Kinsella and Glick, 1953; Baggett *et al.*, 1953).

The basic observation which led to the utilization of this enzyme was made more than two decades ago by Cohen and Marrian (1935). They found that there was complete hydrolysis of urinary estrogen conjugates in human pregnancy urine if conditions conducive to certain bacterial growth were favorable. Since this original observation, many investigators have reported the conditions necessary for enzyme hydrolysis of urine and these were eventually applied to the hydrolysis of blood plasma.

Bayliss (1952) studied conditions which would yield maximum amounts of adrenocorticosteroids as determined by the liberation of formaldehyde. Acidification to a pH of 4.5, buffering with 1.0 *M* acetate, was found to be optimal for urine. Finally, 5000 units of rabbit liver glucuronidase are added per 100 ml. of urine, and the mixture is incubated for 48 hours at 37°C. This pH, and temperature, also leads in all likelihood to the release of a conjugate which has been found by Paterson and Marrian (1951) and Cox and Marrian (1951) to be chloroform soluble but hydrolyzable and stable at pH 4.5. Subsequently, the urine is extracted, acidified further to a pH of 1.0, and left at room temperature for 24 hours before it is extracted again. This final treatment will liberate the free compounds which are presumably not glucosiduronides but which are slowly hydrolyzed by acid (Paterson and Marrian, 1951). In addition, it is essential that the urine be hydrolyzed at pH 2.0 if significant amounts of aldosterone are to be extracted (Luetscher, 1956; Dryrenfurth and Venning, 1957; Ayres *et al.*, 1957a; Neher, 1959).

The properties of different enzyme preparations have been shown to vary. Thus, Buehler *et al.* (1949) showed that the pH requirement of  $\beta$ -glucuronidase of bacterial origin was near neutrality while the pH optimum for splenic glucuronidase was found to be 4.0–4.5 (Cohen, 1951;

Dustan *et al.*, 1953). The concentration of the  $\beta$ -glucuronidases necessary for optimal hydrolysis have not been clearly defined and, in fact, much confusion surrounds this particular point. The rate of hydrolysis is apparently not directly related to enzyme concentration since there are many inhibitory factors in urine which prevent the action of  $\beta$ -glucuronidases. For instance, Bayliss (1952) indicated that the requirement for large amounts of enzyme (much larger than those she designated) are necessary to overcome initial inhibition by the hydrolytic products (i.e., the free corticoids released). This suggests that the most adequate method for obtaining quantitative amounts of steroids would be one using a combination hydrolysis and continuous extraction method. The above findings are analogous to those presented by Karaunairatnam and Levvy (1949) where the liberated glucuronic acid causes inhibition of the  $\beta$ -glucuronidase and of those findings reported by Dustan *et al.* (1953) and Golub *et al.* (1958) where other inhibitory factors are present in urine. Other inhibitors of  $\beta$ -glucuronidase preparations are known. Thus, bacterial  $\beta$ -glucuronidase is inhibited by ammonium ions (Buehler *et al.*, 1951) and saccharate has been shown to be a specific inhibitor of ox spleen glucuronidase (Karaunairatnam and Levvy, 1949).

As in the case of urine, steroid conjugates have been shown to be present in blood (Bongiovanni *et al.*, 1954; Bongiovanni, 1954; Bongiovanni and Eberlein, 1955; Klein *et al.*, 1955; Reddy *et al.*, 1955; Weichselbaum and Margraf, 1955; Vermeulen, 1956). It has been mentioned also (see above) that the ratio of free steroid to conjugated steroid in the blood may fluctuate according to the state of the individual. Consequently, the use of hydrolytic procedures is necessary to obtain a complete picture of corticoid output as reflected by blood plasma and urinary levels.

Few methods have been reported for the hydrolysis of steroid conjugates in blood although it is generally assumed that optimal conditions for urine hydrolysis are applicable to blood. Methods have been proposed by Bongiovanni and Eberlein (1955) and Szego (1957) for total adrenocorticosteroid assay in blood. The method of the former group, described originally for plasma, has been used more extensively. The plasma proteins are first precipitated with 95% ethanol (2.5 volume). No appreciable loss of adrenal steroids appears to take place by adsorption on precipitated plasma proteins since proteolytic enzyme hydrolysis of the precipitated proteins yielded only traces of reacting steroid-like material. After centrifugation and removal of the proteins and most of the alcohol by evaporation the aqueous phase is made up to its original volume with water. The free steroids are obtained by direct extraction of the aqueous phase. The conjugated steroids are obtained by hydrolyzing the aqueous

residue and re-extracting. In this manner a measure of the free and conjugated adrenocorticosteroid (total corticosteroids) may be obtained.

Recovery data of added tetrahydrocortisone monoglucosiduronidate (0.70–10.0  $\mu$ g.) to 10 ml. of plasma ranged from 80–105% of the added material. On the other hand, recovery experiments using mineral acid (pH 1) for hydrolysis did not lead to significant recoveries and there appeared to have been almost complete destruction of the steroid at this hydrogen ion concentration. This again clearly illustrates that acid hydrolysis even under relatively mild conditions must be used with great care.

It is not known whether  $\beta$ -glucuronidase hydrolysis of aqueous homogenates of tissue would lead to a more complete extraction of adrenal steroids. Troen (1959) has presented evidence that placenta perfusate must be subjected to  $\beta$ -glucuronidase hydrolysis in order to obtain all the Porter-Silber-reacting material. This datum indicates that the adrenocorticosteroids in the placenta and other tissues may exist as glucosiduronidate complexes.

*c. Solvolysis.* Because of the sensitivity of some steroid sulfates to hot mineral acids, milder methods for their hydrolysis have consistently been sought. In an extension of the work of Cohen and Oneson (1953), Burstein and Lieberman (1958a, b) described a mild procedure for the splitting of urinary 17-ketosteroid sulfates. It involves solvolysis, i.e., cleavage of the sulfate-steroid bond in organic solvents. The nature of the solvent determines the rate of solvolysis which decreases with increasing polarity of the solvent. As a result of these studies Burstein and Lieberman (1960) have applied their method to the determination of androsterone and dehydroisoandrosterone in plasma with great success. However, these procedures cannot be used for the determination of adrenocorticosteroids since the process of solvolysis does not cleave steroid phosphate or glucosiduronidates. It is conceivable that conditions will be found whereby adrenal steroid conjugates can be hydrolyzed by solvolysis.

## 2. Solvents

*a. Kinds of Solvents.* Choice of solvents is based on ability to extract quantitatively the adrenocorticosteroids from urine, tissue, or blood. The use of ethyl ether for extraction is not recommended because of its high volatility and explosiveness. In addition unless it is purified and used fresh it subjects the extract to the action of peroxides which readily form in ethyl ether unless special precautions are taken for its storage. Furthermore, not all corticosteroids are soluble in this solvent. The last objection is overcome by using various mixtures of chloroform-ether (Heard

*et al.*, 1946; Nelson and Samuels, 1952) and alcohol-ether (Corcoran and Page, 1948; Butt *et al.*, 1951).

Chloroform as a solvent has been used extensively as well as ethylene dichloride and methylene dichloride. Unless the extraction is carried out very carefully, these solvents will usually give rise to emulsions which are very stable and difficult to break (Hechter, 1949; Reich *et al.*, 1950). These can be avoided to a certain degree by adding fairly large amounts of the solvents to the liquid extracted in a ratio of about 2:1 or 3:1. Methylene dichloride is the choice solvent in the group of the chlorinated hydrocarbons because it remains stable, once it is adequately purified, for several months at room temperature (Peterson *et al.*, 1957). Ethylene dichloride and chloroform will remain in a usable state for only a few days.

Ethyl acetate has been shown to have excellent solvent properties toward corticosteroids. Meyer (1953) after testing several solvents for the extraction of steroids in blood found this solvent very satisfactory from this viewpoint; in addition, it extracted half the amount of extraneous nonsteroidal material in the form of pigment, etc., as compared to chloroform or chloroform-ether mixtures; and, it is less prone to form emulsions with urine (Burstein and Dorfman, 1954, 1955; Burstein *et al.*, 1955; Péron and Dorfman, 1956). Furthermore, Burstein (1956) showed that the  $C_{21}O_5$  and  $C_{21}O_6$  steroids had significantly greater partition coefficients in ethyl acetate-aqueous systems than in corresponding chloroform systems. This was also found to be true by Peterson *et al.* (1957). Romanoff *et al.* (1957) showed that the efficiency in extracting Porter-Silber-reacting material increased progressively as follows: ether-chloroform 1:3, methylene dichloride, isopropyl acetate, ethyl acetate. The ethyl acetate and the isopropyl acetate had the further advantage of extracting less urinary pigments which resulted in lower blank values when colorimetric methods of assay were used. The authors have also been able to obtain consistently the highly polar compound  $\beta$ -cortolone from urine (Romanoff *et al.*, 1959) by using ethyl acetate thereby confirming the findings of Burstein (1956) that  $C_{21}O_6$  steroids are easily extracted with ethyl acetate. In certain instances, ethyl acetate cannot be used since it is miscible with the reagents used in some assay methods. In these cases methylene dichloride is used as a substitute since it has excellent solvent properties (Peterson *et al.*, 1957).

Thus far, the solvents discussed are ones used for the extraction of free steroids and those liberated in their solvent-soluble form following  $\beta$ -glucuronidase hydrolysis. Dioxane has been used for the extraction of estrogen sulfates (Grant and Beall, 1950) and 17-ketosteroid sulfates

(Cohen and Oneson, 1953) whereas butanol has been used to extract adrenocorticosteroid conjugates from urine and blood (Reddy *et al.*, 1952, 1956; Forsham, 1954; Reddy, 1954; Wolfson, 1954; Schneider and Lewbart, 1956, 1959). Reddy (1954) and Reddy *et al.* (1956) have described a procedure whereby the free and conjugated corticosteroids are extracted from urine and plasma with butanol. However, high blank values occur with this extraction procedure. In the analysis of some urine extracts the actual color due to the measured  $17\alpha$ -hydroxycorticosteroids represented only a small percentage of the total. Therefore, the applicability of such a method would be questionable when small concentrations of steroids are to be measured. These authors adjust the urine or plasma to pH 1 prior to extraction, whereas Forsham (1954) has recommended bringing urine to a pH of 2.5 to decrease the extraction of nonspecific material and to obtain low blanks. A higher pH (3.5) resulted in loss or nonextractability of Porter-Silber material. Butanol extraction of small samples of plasma (after precipitating the proteins with zinc sulfate) to which had been added 5–20  $\mu$ g. of free adrenal steroids led to recoveries which ranged from 94 to 110% (Reddy *et al.*, 1956). The recovery was 92–106% when 4–36  $\mu$ g. of the conjugated derivative was added.

The procedure one selects for extracting steroids either as free or conjugated forms for analytical purposes is purely a matter of choice and of understanding the limitations and problems involved. The free forms are more desirable than the conjugated since, as was mentioned earlier, present methods for separation of adrenal steroid conjugates have not been adequately developed for quantitative studies (Schneider and Lewbart, 1956, 1959).

There is ample evidence which shows that blood adrenocorticosteroids are "bound" in some fashion to plasma proteins (Szego and Roberts, 1953; Eik-Nes *et al.*, 1954a, b; Daughaday, 1955, 1957). Fortunately this complex is a weak one and does not present a problem in the extraction of adrenocorticosteroids since it can be broken by the simple process of osmolysis (Savard, 1957) and is easily dissociable at room temperature with organic solvents (Folch-Pi, 1953). In addition, solvents which cause denaturation (ethanol, acetone) or those which are mild (ethyl acetate) can extract adrenal corticosteroids from blood or blood plasma equally well (Bush, 1957).

Extraction of corticosteroids from tissues for purposes of chemical assay has not been a common practice because very few of these methods are sufficiently specific or sensitive to make detection possible at the concentrations found in tissues. Ethanol (33%) has been found satisfactory to extract corticosterone from rat and mice adrenals (Silber *et al.*,

1958; Péron and Dorfman, 1958; Halberg *et al.*, 1959; Moncloa *et al.*, 1959). The ethanol serves a dual purpose, it precipitates most proteins and extracts the corticosterone. The latter is then easily extracted from the aqueous ethanol with methylene dichloride.

In other studies, Ncher (1958) first homogenized human adrenal glands in saline and followed this by the precipitation of the proteins with 10–20 volumes of acetone. After removal of the residue and acetone, the aqueous solution is extracted with chloroform. This method is similar to the cold acetone method procedure used by Szego (1957) for the extraction of blood plasma.

*b. Emulsions.* The advances in technique and the greatly increased sensitivity of the existing chemical assay methods for measuring corticosteroid in urine, tissue, or blood have made the problem of emulsions a secondary one. Usually the size or the volume of the samples one is dealing with are such that if emulsions form at any of the extraction or washing stages, they can be broken easily by direct centrifugation in the extracting flask or tube. On the other hand, when large volumes of urine, plasma, or tissue (as a homogenate) are extracted in order to isolate and quantitate individual steroids the emulsion problem becomes more difficult to handle.

To circumvent the problem of emulsions in blood, some trial methods were based on adsorption procedures with charcoal (Levy *et al.*, 1953), dialysis against aqueous media (Hechter, 1949), with a continuous countercurrent dialyzer (Nelson and Samuels, 1952), and dialysis against a two-phase methanol-chloroform system (Zaffaroni, 1953). However, because of poor recoveries, artifact formation, lengthy periods for equilibration, and large volumes of dialyzate to be re-extracted these methods are not applicable to routine use. To break emulsions, Mason (1954) has suggested drawing off the emulsion and adding to it the subsequent 3 to 4 extracts. By the time the fourth extraction has been added, the emulsion usually breaks. This procedure is equivalent to adding large amounts of solvents to the original aqueous medium which is being extracted.

Bradosol ( $\beta$ -phenoxyethyltrimethylammonium bromide, 5% solution, Ciba Laboratories Ltd.) inhibits emulsion formation at a concentration of 1 ml. per liter of urine. It has been successfully used by Layne and Marrian (1958) in the isolation of  $16\alpha$ -hydroxy estrogens from urine. However, its use is contraindicated when some of the chemical assays are applied to relatively crude urine extracts. Romanoff (1957) has found a two- to threefold increase in the optical density (410  $m\mu$ ) of blanks (Porter-Silber) compared to those when Bradosol was absent from extracted urine. In addition these blanks were purple and highly pigmented.

A similar effect was found when the assays were carried out with a blue tetrazolium reaction. In this case, the blanks were 40% higher than those run in the usual manner.

Ethyl acetate can be used for large extractions with advantage. Not only has it a high partition coefficient compared with other solvents (see Section II, C, 2, a), but it does not cause emulsions (Bush, 1952, 1953; Simpson *et al.*, 1952; Meyer, 1953; Burstein and Dorfman, 1954; Haynes *et al.*, 1954; Péron and Dorfman, 1956; Romanoff *et al.*, 1957). In addition, it is a solvent which is not too volatile, is easily obtained, and is reasonable in cost. The extraction of blood corticosteroids may be carried out directly with ethyl acetate (Meyer, 1953) or by first precipitating the proteins with ethanol and then re-extracting the aqueous phase after removal of the ethanol with dichloromethane according to the method of Bongiovanni and Eberlein (1955). In this manner, the proteins which give rise to the troublesome emulsions have been removed. Tissues after homogenization in water may be extracted similarly with advantage.

*c. Hand Extraction versus Continuous Extraction.* Hand extraction has been by far the most popular method of extracting urine because it is simple and inexpensive. Usually 4 to 5 extractions by hand with a solvent (50–100% by volume) having a high degree of extractability (ethyl acetate, isopropyl acetate, or methylene dichloride) is sufficient to yield quantitative recoveries. The problems encountered with a continuous extractor are many, one of the most serious being that it does not guarantee an emulsion-free extract. These form, in spite of a gravity or lighter-than-water feed of solvent. Secondly, the solvent will extract any acids which are found in the urine and deliver these to the boiling flask. There they are concentrated and even at the low boiling temperature can possibly lead to some adrenocorticosteroid destruction. A continuous extractor which operates without distillation of the extracting solvent has been described by Cohen (1950) which overcomes some of the failings outlined above. On the other hand, Appleby and Norymberski (1955) did not find a cascade extractor (Kies and Davies, 1951) satisfactory for the complete extraction of urinary 21-deoxycorticosteroids. Unless there are very large volumes or a large number of samples of urine to be extracted there is no apparent advantage in using a continuous extractor in favor of the time-proven, hand-extraction method.

### 3. Purification of Extracts

Once the problems of hydrolysis and extraction have been solved one should be in a position to assay the extract by the various chemical assay methods available. However, in order to obtain values which can be in-

telligently interpreted, these crude extracts must be purified further to eliminate as much interfering material as possible.

*a. Sodium Hydroxide.* One feature common to most extraction procedures for adrenocorticosteroids, regardless of the solvent used, is the alkali wash (usually 0.1 *N* NaOH) given the extract prior to further purification. This step removes most of the acids, estrogens, and much pigmented material which can interfere in the chemical assays of adrenocorticosteroids. It must be carried out quickly with a volume of the alkali which is small relative to that of the extract and preferably ice cold. If carried out in this manner, very little loss in corticosteroids is sustained in spite of the evidence that there can be destruction of small amounts of adrenal steroids (Venning, 1954). This minor loss of steroid does not warrant omitting the alkali washing step for purification purposes. Of more importance, however, is an awareness of the fact that losses of certain specific steroids, exemplified by  $\beta$ -cortolone, can occur into the "phenolic" fractions of urinary extracts (Fukushima *et al.*, 1955). Recently Romanoff *et al.* (1959) showed that 0.1 *N* sodium hydroxide does indeed extract  $\beta$ -cortolone and that as much as 22% of the latter was found in the alkali washes of ethyl acetate extracts of urine. Whereas 98–108% of tetrahydrocortisol and cortisone added to urine was recovered in alkali-washed ethyl acetate extracts of the urine (Romanoff *et al.*, 1957) only 80% of  $\beta$ -cortolone was recovered in the combined ethyl acetate extracts of the urine, the wash water, and the alkali washes. This indicates that possibly some destruction of  $\beta$ -cortolone occurred. It is probable that the site of this destruction is in the alkali wash prior to acidification and re-extraction with ethyl acetate. It is not known at present whether other steroids are extracted from ethyl acetate with 0.1 *N* sodium hydroxide.

*b. Simple Liquid Partitioning.* Other procedures in combination with the alkali wash will sometimes help to partially remove impurities. These involve the judicious use of washes and extraction solvents (Silber and Porter, 1957) which can be used in conditions like bilirubinuria or when urine is colored with metabolic products of certain foods or drugs. In such cases, Gornall and MacDonald (1953) have found that a benzene-water partition results in more accurate values. This relatively simple procedure which is based on a method reported by Talbot *et al.* (1945) has led to very poor recoveries of pure corticosterone and 11-dehydrocorticosterone. For this reason it is not recommended for quantitative work when the presence of relatively large amounts of these substances are anticipated. However, it can be used when small amounts of cortisol or other highly hydroxylated steroids are to be extracted (Zimmermann, 1955). Eik-Nes (1957) used a benzene-water partitioning of chloroform extracts of blood which when subsequently chromatographed on a Florisil column gave



good maxima at 410  $m\mu$  with the Porter-Silber reagent. In addition, he showed that when 0.10–1.6  $\mu\text{g.}$  of cortisol were added to blood plasma and subjected to partition and chromatographic procedures 94–100% recoveries were obtained with very good reproducibility.

Another very common and popular method which is especially applicable to fatty extracts obtained from blood plasma or tissues is based on the fact that aqueous solutions of adrenal steroids can be easily and safely partitioned against petroleum ether (ligroin). The extract is dissolved in 70% ethanol or methanol and then partitioned with petroleum ether. Most lipids will dissolve in the petroleum ether resulting in an almost fat-free aqueous ethanolic phase. The ethanol is evaporated and the aqueous residue is reconstituted with water to the original volume. The aqueous phase is extracted with a suitable solvent and washed with alkali. This procedure yields in many instances an extract which is sufficiently purified for the direct application of chemical assays.

Blood plasma or tissue (as a homogenate) also may be extracted directly with petroleum ether followed by extraction with a suitable solvent which leads to a "fat-free" extract. Instead of the ligroin partition, carbon tetrachloride has been used with similar results (Silber and Busch, 1956; Morris and Williams, 1953). The decision to use carbon tetrachloride or ligroin depends on the volume of the sample to be extracted and whether it is more convenient to have a solvent which is heavier or lighter than the sample. But, carbon tetrachloride like methylene dichloride has a tendency to form stable emulsions which can be broken only with difficulty. However, as has been mentioned above, this offers no real problem in the usual microchemical assays since emulsions can be broken by centrifugation.

*c. Column Chromatography.* Further purification of extracts can be the subject of relatively simple column adsorption or partition chromatography, rather than paper chromatography which is usually used for the separation of a multicomponent steroid mixture in a purified extract. In these instances the columns are used to eliminate nonsteroidal material which interferes but does not appreciably resolve the steroids in the mixture. Usually the conditions are established so that the nonsteroidal material is eluted first and the adrenal steroids eluted in subsequent fractions. Some of the adsorbents used to prepare such columns are excellent in spite of the fact that they lack resolving power. Silica gel (silicon dioxide) and Florisil (magnesium silicate) are examples of two commonly used adsorbents whereas Celite (diatomaceous silica products) has been used more often for preparing partition columns with high resolving properties.

No special precautions need be taken in preparing the silica gel col-

umns and the material may be used as obtained from the manufacturer (Levy *et al.*, 1953). It can be activated by mixing in small volumes of water which results in an increase in its resolving power. Sweat and Farrell (1952) have successfully measured plasma corticosteroids fluorometrically by purifying the plasma extracts by micro silica gel chromatography. Recoveries of standard cortisol and corticosterone on such columns were found to be 93–99% and 90–101%, respectively.

Romanoff *et al.* (1953; Romanoff and Wolf, 1954) have applied silica gel chromatography to extracts of large volumes of human urine in their extensive investigations. The residues obtained from methylene dichloride extracts of urine were placed on columns from which the steroids were eluted and separated into three major steroid fractions. The first and least polar-eluting solvent (benzene and benzene-ether 2:1) removes most of the pigments, waxes, and fats. The two fractions removed approximately 70% of the weight of the original extract which is almost all nonsteroidal material. This makes possible the assay of the subsequent fractions which would have been difficult prior to chromatography. This method was also used by Péron and Dorfman (1956) as a preliminary purification step prior to the separation of adrenocorticosteroids and 17-ketosteroids in ethyl acetate extracts of guinea pig urine.

Florisil columns, which were introduced by Nelson and Samuels, (1952) have had more extensive use for routine chemical assays on small volumes of urine and blood. With this technique, 17-ketosteroids can be separated from corticosteroids. The method has been shown to have a high degree of reproducibility, and recoveries of cortisol from urine after  $\beta$ -glucuronidase were 92–96% (Glenn and Nelson, 1953).

Recovery values of cortisol (1–4  $\mu$ g.) added to 30.0 ml. of blood were more variable (80–112%). Therefore, it is advisable to obtain recovery values when using the Florisil column for purposes of purification of blood plasma extracts (Nelson and Samuels, 1952). Bush and Sandberg (1953) and Migeon *et al.* (1956b) found that values obtained from plasma extracts purified on Florisil columns were correlated with those obtained after paper chromatography. Unlike silica gel, certain precautionary measures must be taken for the successful use of Florisil (Eik-Nes *et al.*, 1953). It has to be washed carefully beforehand with absolute ethanol, dried, and then activated at 600°C. in a muffle furnace. The Florisil so prepared must be kept stored in a desiccator especially when the humidity is high. Occasionally, the use of Florisil columns has resulted in high blanks due to residues eluted from the Florisil itself. This leads to considerable variation in results and for this reason it is advisable to discard the Florisil and use another lot preparation.

### III. Methods of Assay

#### A. GENERAL

In the previous section, considerable space has been devoted to what may be called the foundation upon which the chemical assays are built. Unless accurate and sufficiently simple methods for purifying extracts of urine, tissue, or blood are available and applicable to routine work, little can be gained by developing new superstructures in the form of new and more sensitive chemical assays. Once clean extracts are obtained by procedures similar to those outlined above, they can be either assayed directly or if an analysis of the individual steroid component is desired the extracts can be resolved further by paper chromatographic techniques.

The chemical reagents usually used in the chemical assays react with adrenocorticosteroids by virtue of reactive grouping(s) on the steroid nucleus. The resulting reactions can give rise to the formation of colored complexes, the liberation of volatile products, the formation by transformation of new steroid products, and the formation of fluorescent substances (Fig. 1). These can all be measured with relative simplicity and accuracy with the methods which are presently available.

Group 1 depends on the reducing properties of the  $\alpha$ -ketolic side chain which will give rise to colored complexes after the addition of certain reagents. The reagent used most commonly in this type of reaction is blue tetrazolium (BT, 3,3'-dianisole bis [4,4'-(3,5-diphenyl)tetrazolium chloride] which forms a deep-blue-colored complex. Group 2 depicts the ability of periodate to oxidize and cleave the side chain of certain adrenal steroids. The products of the reaction, 17-ketosteroids, formaldehyde, or acetaldehyde are measured by standard Zimmermann (see Chapter 2) and chromotropic acid reagents, respectively. Sodium bismuthate is used in group 3 instead of periodate, the bismuthate oxidizing the side chain of certain 17 $\alpha$ -hydroxy-C<sub>21</sub> steroids to 17-ketosteroids.

The reaction given by group 4 with a mixture of phenylhydrazine sulfuric acid forms the basis of a method for measuring 17 $\alpha$ -hydroxy, 20-keto, 21-hydroxy steroids specifically. Their measurement depend on the formation of a colored 3,20- or 3,21-bisphenylhydrazone (Silber and Porter, 1957) which has a maximum absorption in the 410-m $\mu$  region of the spectrum.

In other methods certain adrenal steroids will react rather specifically with sulfuric or phosphoric acid to give rise to fluorescent light when activated by monochromatic light between 200 to 800 m $\mu$ .

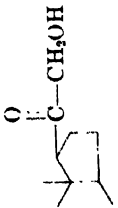
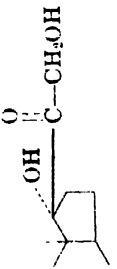
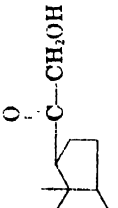

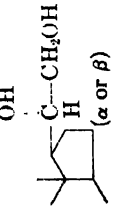
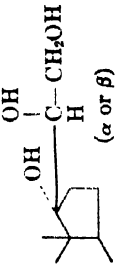


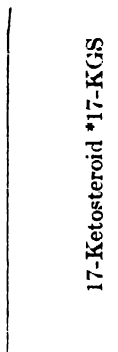


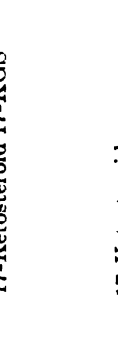
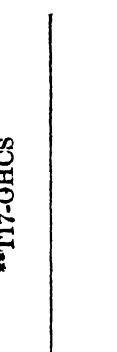
Ring D side chain of steroids measured	Reagent used	Product of reaction	Product which is measured colorimetrically
<b>GROUP 1</b>			
(a) 	Blue tetrazolium	Formazan	Formazan
(b) 	Blue tetrazolium	Formazan	Formazan
<b>GROUP 2</b>			
(a) 	Periodic acid	C <sub>30</sub> acid + formaldehyde	Formaldehyde
(b) 	Periodic acid	C <sub>30</sub> acid + formaldehyde	Formaldehyde
(c) 	Periodic acid	C <sub>30</sub> acid + formaldehyde	Formaldehyde
(d) 	Periodic acid	17-Ketosteroid + C <sub>2</sub> acid	17-Ketosteroid

Fig. 1. Typical side-chain groupings which react with chemical reagents used in chemical assays. \*17KGS, 17-ketogenic, \*\*T17-OHCS, total 17-hydroxycorticosteroids.

 <p>(e)</p>	Periodic acid	Ring D acid? + acetaldehyde	Acetaldehyde
 <p>(f)</p>	Periodic acid	17-Ketosteroid + acetaldehyde	17-Ketosteroids and acetaldehyde
GROUP 3			
 <p>(a)</p>	Sodium bismuthate	17-Ketosteroid + C <sub>2</sub> acid?	17-Ketosteroid *17-KGS
 <p>(b)</p>	Sodium bismuthate	17-Ketosteroid + C <sub>2</sub> acid?	17-Ketosteroid 17-KGS
 <p>(c)</p>	Sodium bismuthate	17-Ketosteroid + C <sub>2</sub> acid?	17-Ketosteroid 17-KGS
 <p>a, b, c, and (d)</p>	Sodium borohydride followed by sodium bismuthate	17-Ketosteroid + C <sub>2</sub> acids	17-Ketosteroid **T17-OHCS
GROUP 4			
 <p>(a)</p>	Phenyldiazine hydrochloride	3,20- or 3,21-Bisphenylhydrazine zone	Hydrazine

Radioisotopic methods have also been utilized in conjunction with paper chromatography to determine levels of certain adrenal steroids in urine and tissue. Although the actual method of assessing the radioactivity is a physical rather than a chemical one, nevertheless quantitation of the steroids eluted from the chromatograms is ascertained by chemical assay.

In studies concerned with adrenal function, the corticosteroids and their metabolites possessing the C-20,21- $\alpha$ -ketolic side chain are the steroids most commonly measured by chemical assays. This is not surprising since they account for approximately 50% of those elaborated by the adrenal gland. However, as was mentioned earlier, methods for the measurement of 21-deoxy steroids specifically should be available in order to enable a diagnostic differentiation of several conditions causing adrenal hyperplasia.

The chemical methods which will be described below are representative of the reactions discussed above. Before each outline of the chemical procedures which are given at the end of each section in chemical methods, their applicability to urine, blood, or tissue extracts is discussed from the time of their inception up to the present day. Thus, the requisites for understanding a particular chemical assay will have been discussed and outlined.

## B. CHEMICAL METHODS

### 1. *Blue Tetrazolium*

Since many nonsteroidal  $\alpha,\beta$ -unsaturated ketones present in urine extracts were found to cause reduction of phosphomolybdic acid (Heard and Sobel, 1946), methods were devised which depended on the reducing properties of the C-20,21- $\alpha$ -ketolic side chain of adrenocorticosteroids. Highly variable results were also obtained because the reducing powers and rates of reduction of some steroids varied greatly with the phosphomolybdic acid reagent.

In a procedure using the blue tetrazolium salt which is reduced to the blue formazan by  $\alpha$ -ketols (20-keto,21-hydroxy) Chen and Tewell (1951) developed a workable procedure which necessitated the preparation of a blank especially when colored urinary extracts were analyzed. In the following year, Henly (1952) proposed using the slightly less intense red color obtained by neutralization of the blue color produced by 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride instead of the blue tetrazolium utilized by the afore-mentioned workers. The method was reported to be more sensitive than the BT reaction. However, be-

cause the former compound is unavailable, it has had no application in the determination of adrenal steroids in biological fluids.

Mader and Buck (1952) in developing a procedure for the control assay of adrenal steroids carried out their reaction in tetramethylammonium hydroxide instead of sodium hydroxide (Chen and Tewell, 1951). Their method was specific for steroids having a reducing  $\alpha$ -ketolic side chain. However, after 35 minutes when all the steroids tested had reached their maximum color cortisone acetate and 11-deoxycortisol acetate produced less color than three  $\alpha$ -ketolic steroids without the 17-hydroxy group. It appeared, therefore, that the presence of a 17 $\alpha$ -hydroxy group in adrenal steroids affected the rate of color development by BT. Two sensitive methods capable of measuring 5  $\mu$ g. of reducing steroid by BT were presented by Chen *et al.* (1953). In one, sodium hydroxide was used as the alkaline reagent, and choline in the other. Both methods showed good agreement when applied to chloroform extracts of human urine (equivalent to 20 ml. of urine). Although  $\alpha,\beta$ -unsaturated 3-ketones react with the BT reagent, they are unlikely to interfere especially at the concentrations found in biological fluids and tissue. Other substances which react to a considerable degree with the BT reagent such as catechol amines and 5-hydroxyindoles (Rosenkrantz, 1959) would remain in the aqueous phase because of their high solubility in water.

Weichselbaum and Margraf (1955) extensively and carefully investigated total reducing plasma steroids by applying the method of Mader and Buck (1952). They found that plasma extracts purified initially by column chromatography were suitable for measuring BT-reducing steroids. The validity and sensitivity of the methods were checked with established methods.

Recknagel and Litteria (1956) found that the BT method suffered from lack of reproducibility in the degree of color development; they believed this was due to the poor quality of the base (tetramethylammonium hydroxide) used. To obtain more consistent color development, they proposed using sodium hydroxide, at a final normality of  $3.33 \times 10^{-2}$  to develop the color, at room temperature. Although strict adherence to the Beer-Lambert law was maintained, the method suffered as in the original methods of Mader and Buck (1952) and Chen *et al.* (1953), in that optical density values significantly varied with the adrenal steroid measured. This occurred even at relatively low concentrations.

Incorporating the failures and successes of the above authors as a background for further studies, Izzo *et al.* (1957) were able to determine conditions which minimized the optical density values contributed by nonketolic steroids. With their modification equal color is achieved

with all ketolic steroids tested without sacrificing the sensitivity of the method. If this method is used in the determination of  $\alpha$ -ketolic steroids in the presence of nonketolic steroids, a correction is applied to the 30-minute optical density readings in order to obtain a reading which is due to the  $\alpha$ -ketolic steroid alone. Thus, the increment in optical density during the second 30 minutes after initiation of the BT reaction, which is due to non- $\alpha$ -ketolic steroids, is subtracted from the optical density at 30 minutes. This is justifiable only if all  $\alpha$ -ketolic steroids have reacted after 30 minutes and if the color contribution made by nonketolic steroids is a straight-line function of time up to 60 minutes. However, such a correction would not guarantee that nonsteroidal BT-reducing material in a biological extract does not react with the same intensity and speed as the known  $\alpha$ -ketolic steroids (Rosenkrantz, 1959).

A modification of the BT method outlined by Elliott *et al.* (1954) has been used in the author's laboratory for measuring the production of corticosteroids of rat adrenal slices or homogenates incubated *in vitro* (Koritz and Péron, 1958; Péron and Koritz, 1958, 1960). Cortisol and corticosterone were the only steroids used as reference standards, but they gave nearly the same color intensities with the conditions to be outlined (see p. 223). The method is highly sensitive and capable of measuring accurately 1  $\mu$ g. of cortisol or corticosterone and detecting as little as 0.4  $\mu$ g. of these substances. The values obtained for the blanks (0.02) and samples (2  $\mu$ g. = 0.068) are duplicable from day to day. In fact, although standards are always included when the chemical assay of rat adrenal steroids is carried out, the daily and individual curves have been almost identical over a 4-year period.

In a study designed to accentuate the difference in reducing power between various steroids Meyer and Lindberg (1955) reported the conditions required to obtain maximum specificity of BT toward primary  $\alpha$ -ketols. They indicated that this occurred at a minimum basicity of 0.0025 *N* with tetramethylammonium hydroxide at a temperature of 40°C. for 15 minutes. When the conditions for the reaction are modified with respect to time, temperature, and normality of the base(s) other steroids will reduce BT (Meyer and Lindberg, 1955; Touchstone and Hsu, 1955; Izzo *et al.*, 1957). In addition, the presence of nonsteroidal substances, which will also interfere with the specificity of the BT methods, are to be anticipated in biological material.

Reducing sugars, glycols, (un)saturated fatty acids, and waxes are but a few of these substances. However, the water-soluble substances are not extracted into the organic solvent and the lipid material can be readily removed by any one of the several purification procedures discussed above. In spite of these, other BT-reducing products may



still remain in the final extract and will reduce the specificity of the method and lower its sensitivity. Despite a low specificity in this respect, the BT methods have shown their usefulness as routine reactions for clinical purposes (Vermeulen, 1957).

These conclusions are further substantiated by the findings of Izzo and Keutmann (1957). They found that the sum of the reducing values of all chromatographic fractions obtained by the resolution of a neutral purified extract of urine on a paper chromatogram agreed well with the total reducing value of the original extract. The  $\alpha$ -ketols isolated on the paper accounted for 80 to 95% of the total reducing value of the urines studied. As a further test of the method, it was shown that after the administration of cortisol the increase in total BT-reducing value of the urine was accounted for completely by the increase in identifiable reducing  $\alpha$ -ketolic substances isolated on the chromatograms. Therefore, in general the total BT-reducing value of a purified urine extract may be used as an index of adrenocortical activity. However, caution must always be exercised in interpreting results obtained in certain cases, for example, in virilizing adrenal hyperplasia. In such cases, a normal BT titer is obtained from neutral extracts of urine which cannot be accounted for as steroidal material on paper chromatograms run in conventional systems (Eberlein and Bongiovanni, 1955). Marks *et al.* (1957) have also found a very low level of specificity of the BT reaction and a lack of correlation with the Porter-Silber reaction. This was based on findings obtained with chromatographed (Florisol) neutral extracts of normal and pathological urines. On the other hand, the recent work of Jensen (1959) does not substantiate the above findings since values obtained with the Mader-Buck procedure were equal to those obtained with the Porter-Silber method.

The BT reaction can be used for the quantitative determination of an  $\alpha$ -ketolic steroid eluted from paper chromatograms. Since small amounts of stationary phase(s) and reducing substances leached from the paper chromatograms reduce the BT reagents, paper blanks of the same area from a part of the chromatogram free of any steroid or pigment, are usually eluted with the same volume of solvent and treated with BT. Alternatively, a blank paper chromatogram may be run at the same time in order to elute nonspecific BT-reducing material from identical areas as those occupied by the steroids on the other chromatograms. The latter procedure is useful when a large number of individual steroids occupy most of the surface area of the chromatogram. However, the correction values so obtained fail to make adequate allowance for other BT-reducing materials in the form of pigments, fatty acids, etc., which may contaminate the steroid zones. Touchstone and Hsu

(1955) published a procedure which took advantage of the water-insoluble nature of the reduced BT color product (formazan). In this way the papers could be washed free of water-soluble, nonspecific, BT-reducing material. The formazan is then eluted quantitatively from the chromatogram by a mixture of ethyl acetate and methanol (7:3 v/v) and read at a wavelength of 550–560 m $\mu$ . In order to obtain maximum precision and accuracy with this method, the amount of  $\alpha$ -ketolic BT-reducing steroid to be applied on the paper chromatograms must be such that no one single chromatographed zone contains less than 20  $\mu$ g. or more than 75  $\mu$ g. of steroid per four-cm.<sup>2</sup> area of paper. With these conditions in 20 duplicate determinations, the average duplication from the mean was found to be 6% with a 97% average recovery when standards were added to urine extracts.

*a. Method of Izzo, Keutmann, and Burton (1957)*

*Reagents*

1. *Blue tetrazolium (BT)*. Dajac grade with a one-degree melting-point range; 0.25% solution in pure ethanol (see reagent 5).

2. *Tetramethylammonium hydroxide (TMAH)*. Ten per cent aqueous solution; 1 ml. of aqueous TMAH in 10 ml. of ethanol. •

3. *Acid alcohol*. A solution of 1 part concentrated hydrochloric acid in 10 parts of ethanol

4. *Steroid solutions*. Pure steroids dissolved in alcohol.

5. *Ethanol*. Redistilled either from (1) *m*-phenylenediamine dihydrochloride (2 gm./100 ml.) or (2) sodium hydroxide pellets followed by refluxing and redistillation in the presence of 2% of reagent in BT or acid alcohol. Reflux 1 liter of alcohol in presence of 7 gm. of metallic sodium and 27.5 gm. of diethyl phthalate for 1 hour followed by distillation.

*Procedure*

1. Accurate aliquots of the standard steroid solutions (or of a purified solvent extract of biological material) transferred to glass-stoppered tubes and brought to dryness under N<sub>2</sub> at 45°C.

2. Addition of 3.0 ml. of the purified ethanol. The author suggests the addition of 3 to 5 drops of methylene dichloride at this step to aid in the solution of fatty materials which may remain in extracts of biological material. This step is also included in the calibration of the standard curve.

3. Addition of 1.0 ml. of 0.25% BT solution.

4. Immersion of the tubes in a water bath kept at 25°C. for 20 minutes.

5. Addition of 1.0 ml. of the dilute TMAH.

6. Addition of 1.0 ml. of acid alcohol after the reaction is completed. This stabilizes the color which will not change over a period of hours.

The reagent blank is prepared by substituting alcohol for the steroid solution. A reverse blank is also carried out by adding 1.0 ml. of the acid alcohol prior to the addition of 1.0 ml. of the TMAH solution. Optical densities are read in a Beckman DU spectrophotometer set at a wavelength of 510 m $\mu$ . Readings are obtained at other wavelengths on either side of the maximum absorption wavelength to ascertain that peaking occurs at, or very close to, 510 m $\mu$ . All samples may be read against alcohol and the proper corrections applied after recording the optical densities. Alternatively, as was proposed by the original authors, the reagent blanks are read against the reverse reagent blanks and the steroid solutions against the reagent blanks.

*b. Method of Elliott, Birmingham, Schally, and Schönbaum (1954)  
as Modified by the Author*

*Reagents*

1. *Absolute ethanol*. Reagent quality.

2. *Blue tetrazolium*. Material obtained from Dajac Laboratories is digested in boiling water and crystallized from a methanol-acetone mixture as recommended by Chen *et al.* (1953). A minimum volume of solvent is used. The purified BT which should be pale yellow in color is stored in a brown bottle in the refrigerator.

3. *Standard solutions*. Cortisol or corticosterone at a concentration of 10  $\mu$ g. per milliliter in absolute ethanol.

4. *Tetramethylammonium hydroxide (TMAH)*. A 10% aqueous solution of TMAH (0.30 ml.) (Eastman Kodak) is diluted to 10 ml. with absolute ethanol. This solution is prepared fresh each day.

5. *Methylene dichloride*. Prepared as outlined by Saffran and Schally (1955). Methylene dichloride is washed with an equal volume of water, dried with anhydrous sulfate, and decolorized with charcoal. The solvent is kept over sodium or potassium hydroxide pellets for 16 hours and then distilled. The fraction boiling at 39°C. is collected.

6. *Blue tetrazolium reagent*. A solution of the purified BT is made up in ethanol (150 mg.%) at room temperature. If complete solution of the material is not obtained the solution is filtered through a fritted glass filter. The clear solution is stored in a brown glass-stoppered bottle. It keeps indefinitely in the cold.

*Procedure*

1. Steroid standards or methylene dichloride extracts of the incubation media of adrenal sections or homogenates are brought to dryness under N<sub>2</sub> at 40°C. in 1.5-  $\times$  6-cm. tubes.

2. The dry residues are dissolved in 0.20 ml. of absolute ethanol. As in the above method 3–5 drops of methylene dichloride are added if there is any visual evidence of a fatty or waxy residue remaining in the tubes. Solution of the residue is hastened by immersing the tubes in boiling water for 5 seconds, shaking, and then bringing to room temperature. If any cloudiness is noticed in the ethanolic phase a few more drops of methylene dichloride are added. Although this solvent does not interfere in the BT assay the same volume of methylene dichloride is added to all tubes including those containing the standards. All tubes are brought to room temperature before the following additions.

3. Blue tetrazolium reagent (0.15 ml.) is added and each tube shaken.

4. Tetramethylammonium hydroxide (0.15 ml.) as in step 3 above is added and each tube shaken.

5. The tubes are stoppered and incubated in a water bath at 30°C. for 20 minutes.

6. Finally, 2.5 ml. of absolute ethanol are added and each tube shaken.

The samples are read in a Beckman DU spectrophotometer at a wavelength setting of 510 m $\mu$ . Readings are recorded after subtracting values obtained from blanks carried through the same procedure. The color developed is stable for a few hours and very little change occurs in the optical densities in 1 hour. It has been a matter of routine in our laboratory to read all samples within 30 minutes. A total of 32 individual determinations may be carried out with ease and accuracy by one operator within a 2-hour period. As was mentioned previously, the sensitivity of the method is such that 0.4  $\mu$ g. of cortisol or corticosterone can be measured. Strict adherence to Beer-Lambert's law is maintained over a concentration range of 0.50 to 15.0  $\mu$ g.

## 2. Periodate Oxidation

Periodic acid oxidations of the side chain of C<sub>21</sub> steroids has been used for quite some time for their characterization and differentiation. The observations of Fieser *et al.* (1944) and Talbot and Eitingon (1944) that certain adrenal C<sub>21</sub> steroids gave rise to 17-ketosteroids when treated with an excess of periodic acid was made the basis of a procedure for their quantitation in urine extracts (Talbot and Eitingon, 1944). Thus, an analysis of the 17-ketosteroid content of aliquots of the extracts prior to and after periodic acid oxidation should have provided a measure of the adrenal steroids excreted. Unfortunately, periodic acid will give rise to 17-ketosteroids only with those steroids which have a glycol or 17 $\alpha$ ,20-dihydroxy-21-methyl type of side chain (group 2, d and f, Fig. 1). Since these represent only a small percentage of the total amount of

urinary steroids known to be derived from the adrenal, this method is limited in its applicability for quantitating adrenal steroids.

On the other hand, it has been used with success by Romanoff *et al.* (1959) for the determination of cortolones eluted from paper chromatograms obtained from urinary extracts of young and elderly subjects. The method was found to be specific, accurate, and sensitive. In determinations of 10 to 30  $\mu\text{g.}$  of  $\beta$ -cortolone by periodic acid oxidation and subsequent Zimmermann determinations, they found, after correcting the values in terms of 11-ketoetiocholanolone equivalents, an average recovery of 99% of the expected 11-ketoetiocholanolone. Recoveries from paper chromatograms were 90% or better at the 50- $\mu\text{g.}$  level.

The above authors have also used the periodic acid oxidation reaction for the detection of 17,20,21-trihydroxy steroids directly on paper chromatograms by the method proposed by E. B. Romanoff and Hunt (1955). The reaction when carried out under aqueous alkaline conditions is specific for 17,20,21-triols whereas when alcoholic acidic conditions are used (Bush, 1955) 17,20-diols also react to a certain extent. However, the acid reagents render the test more sensitive (1-2 versus 5-50  $\mu\text{g.}$  for the alkaline conditions). Both methods require the treatment of control chromatograms with the reagents since some nonsteroidal substances also react to give colored products with the Zimmermann reagent. The control strips also serve to differentiate the colors produced by 3-keto and  $\Delta^4$ -3-ketosteroids from 17-ketosteroids (E. B. Romanoff and Hunt, 1955).

The formaldehyde which is liberated as a result of cleavage of the side chain of C-20,21 ketols by periodic acid (group 2, a, b, and c, Fig. 1) is the basis of another method. The formaldehyde can be measured by the formation of a colored product upon the addition of chromotropic acid directly to the reaction mixture (Lowenstein *et al.*, 1946). To overcome the very high blanks which were obtained, Corcoran and Page (1948) modified this method by distilling the formaldehyde prior to its reaction with the chromotropic acid reagent. The formaldehydogenic steroid values obtained by this method were somewhat lower than those reported by Daughaday *et al.* (1948) yet slightly higher than those of Romanoff *et al.* (1949). Although the latter two groups of investigators had distilled the formaldehyde into a bisulfite solution, the blank values were still very high. Such high values have been attributed to the presence of nonspecific chromogens in the urine extracts which distilled over with the formaldehyde.

In order to eliminate the thermal distillation step, Hollander *et al.* (1951) and Wilson (1953) used Conway cells to obtain an isothermal distillation of the formaldehyde. Distillation of the latter occurred directly into the chromotropic acid reagent. In spite of these changes,

there was no indication that accurate measurements could be obtained nor any assurance that the results could be duplicated. Although nearly complete recoveries of added formaldehyde to urine extracts were obtained in 11 hours of thermal diffusion, Hollander *et al.* (1951) found the recoveries of cortisone and cortisol to be variable. Addition of these substances to ketonic, nonketonic, benzene, and benzene-soluble fractions also led to variable recoveries.

In an extensive investigation concerning these problems, Paterson and Marrian (1953) and Marrian (1954) demonstrated that the poor steroid recoveries were not due to any inhibition of the periodate oxidation reaction. Rather, inhibition was found to be at the point of formaldehyde recovery, and this was probably due to substances which reacted with and retained the formaldehyde during the oxidation. These findings were substantiated by Wilson (1954) and their validity strengthened by the fact that different formaldehydogenic values were obtained by extracting different volumes of the same urine (Mason, 1954).

In an attempt to solve some of these problems, Wilson (1953) and Venning (1954) proposed the use of ethyl ether to extract the inhibiting substances following the oxidation of the steroids with periodic acid. In spite of the obvious drawbacks of the above method of Hollander *et al.* (1951), Engel *et al.* (1955) have used it successfully. They analyzed the distribution of formaldehydogenic lipids in the ketonic fraction of a methylene dichloride extract of urine obtained from a rheumatoid arthritic patient who had received 300 mg. of cortisone. However, if the periodate methods are used and results comparable with other methods are expected as were obtained by Romanoff *et al.* (1949) and Wheeler *et al.* (1953) an almost absolute purification of the organic solvent extracts is necessary. In many cases where values for formaldehydogenic steroids have been reported, this step has not been carried out with any great degree of care. There has been little progress in this direction since Borth (1956) wrote "formaldehydogenic corticosteroids should perhaps be left, for the time being, in the care of the methodologists who are trying to clear up the problems of formaldehyde retention by non-steroidal chromogens."

To obtain a measure of  $C_{21}$  steroid triols and diols in urines, Cox (1952) also used periodic acid to oxidize urine extracts. These steroids (Fig. 1, group 2, e and f) when oxidized with the acid give rise to acetaldehyde instead of formaldehyde. However, the same problems encountered with the formaldehydogenic method appeared to apply to the acetaldehydogenic method, since measurements of the acetaldehyde had to be rigorously standardized. There appeared irregular recoveries of pregnane-

3 $\alpha$ ,17 $\alpha$ ,20( $\alpha$  or  $\beta$ )-triol when this material was added to unwashed chloroform extracts of urine.

With a modification of this method, Cox (1959) has been able to apply it to the determination of the known acetaldehydogenic steroids on paper chromatograms and in urine extracts. The method appears to have the necessary attributes of simplicity, sensitivity, and specificity. What is most striking is the absence of interference from chromogenic substances present in urine extracts. This may be due to the fact that the acetaldehyde is "distilled" with a current of air rather than with displacement by heat. Recovery experiments of small amounts of pregnanetriol, pregnanetriolone, and pregnanetetrol from 25 ml. of urine ranged from 90 to 99%. The specificity of the method has been checked by the mobilities of the known acetaldehydogenic steroids on paper chromatograms and by their characteristic fluorescence with phosphoric acid. In this light, the chemical assay of quantities of 5 $\beta$ -pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol and 5 $\beta$ -pregnane-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,20 $\alpha$ -tetrol which are excreted in the urine of patients with adrenocortical hyperplasia, adrenal tumor, or virilizing adrenal hyperplasia (Butler and Marrian, 1937; Lieberman and Dobriner, 1945; Mason and Kepler, 1945; Finkelstein *et al.*, 1953; Cox and Finkelstein, 1957; Fukushima and Gallagher, 1957b) should prove to be simple as well as accurate.

*a. Periodic Acid Reaction of Talbot and Eitington (1944)*

*Reagents*

1. *Methanol absolute.* C.P.
2. *Periodic acid.* Analytical reagent. Reaction reagent: 14.0 gm. of periodic acid dissolved in 100 ml. of 50% aqueous methanol.
3. *Ethyl ether.* Reagent grade purified by distillation from sulfuric acid after washing with a 5% solution of ferrous sulfate.
4. *Sodium hydroxide.* Analytical reagent; 1 *N* aqueous solution.
5. *Sulfuric acid.* Analytical reagent.
6. *Sodium hydrosulfite.* C.P. reagent; 10 gm. in 100 ml. of 1 *N* aqueous NaOH.

*Preparation of the Urine Extract*

Standard procedures may be used to hydrolyze the urine with  $\beta$ -glucuronidase, i.e., adjusting the urine to a pH of 4 to 6 with acetic acid and buffering with acetate buffer at the same pH. After a 24- to 48-hour hydrolysis period at 37°C. the urine is extracted with ethyl acetate and washed with 1 *N* sodium hydroxide and then water until neutral. The dry extract is separated into a ketonic and nonketonic fraction with the aid of Girard's reagent T (Girard and Sandulesco, 1936), the oxidation being carried out on the nonketonic fraction.

### Procedure

1. The nonketonic fraction (5.0 mg.) is dissolved in 2.7 ml. of methanol.

2. Then 0.1 ml. is removed for 17-ketosteroid analysis (see Chapter 2).

3. The freshly prepared periodic acid reaction reagent (0.5 ml.) is added followed by 0.4 ml. of concentrated sulfuric acid.

4. After standing for 1 hour at 20°–25°C. the solution is extracted with ethyl ether and the ether extract is washed with a 0.10 volume sodium hydrosulfite reagent for 2 minutes.

5. After washing the ethyl ether extract further with 0.10 volume of 1 N sodium hydroxide and then with water until neutral it is evaporated to dryness under reduced pressure.

6. The residue is dissolved in a solvent and aliquots can be taken for the colorimetric assay.

The 17-ketosteroid content of the residues prior to (see step 2 above) and after periodic acid oxidation is determined by the Zimmermann reaction (see Chapter 2). All values should be corrected by a correction equation (Talbot *et al.*, 1942; Allen, 1950). The values obtained after subtracting those for 17-ketosteroids (see step 2) from extracts which have been treated with periodic acid are equal to the 17-ketosteroid content present in the nonketonic fraction.

The application of this method to eluates of paper chromatograms for the analysis of 17,20,21-trihydroxy-steroid glycerols (Romanoff *et al.*, 1959) is carried out by scaling down the volume of all reagents used for the oxidation procedure. No preliminary Girard separation need be carried out. Thoroughly washed Whatman paper No. 540 is used for the chromatograms which are run in the benzene-formamide system of Burton *et al.* (1951). Because of the relative purity of the steroids and the noninterference of other materials which are eluted from the chromatograms (Zimmermann), blanks carried out on eluates not treated with periodic acid give the same values as those obtained from the reagents alone. However, as in the above, a color correction is applied to the Zimmermann values.

A point which should always be considered in reporting 17-ketosteroid values is the standard used for the calibration of the 17-ketosteroid curve. This is especially true where the total extract is used for the oxidation steps, because 11-deoxy, 11-hydroxy, and 11-keto-17-ketosteroids would be measured as a mixture. Since these have different extinction coefficients and differ from that given by dehydroisoandrosterone, a popular standard, the reference standard used must be clearly indicated. Thus, results may be expressed as 11-keto or 11-hydroxyetiocholanolone, 11-



keto or 11-hydroxyandrosterone, androsterone, etiocholanolone, or dehydroisoandrosterone equivalents.

It must be noted that the above method does not permit the quantitation of  $\alpha,\beta$ -unsaturated 3-ketone-17 $\alpha$ ,20,21-glycols (Burstein and Dorfman, 1955). These will have been removed in the ketonic fraction of the Girard separation.

#### *b. Acetaldehydogenic Method of Cox (1959)*

##### *Reagents*

1. *Ethanol and methanol.* Highly purified or refluxed for 8 hours with 2% (by weight) sodium hydroxide and distilled twice.

2. *Sulfuric acid.* Concentrated C.P. grade. Diluted with water to make a 0.4 *N* solution.

3. *Periodic acid.* Laboratory grade. Reagent: 0.14 *M* periodic acid in 0.4 *N* sulfuric acid.

4. *Glycine.* Laboratory grade. Reagent: 1% solution in 0.4 *N* sulfuric acid.

5. *Oxidizing reagent.* A mixture of equal volumes of reagents 3 and 4, freshly prepared.

6. *Air.* Purified by passage through concentrated sulfuric acid then through a soda-lime tower.

7. *Sodium bisulfite.* C.P. grade, 1% aqueous solution.

8. *Copper sulfate.* C.P., 4% aqueous solution.

9. *4-Hydroxydiphenyl.* C.P. grade, 1.5% solution in 0.1 *N* aqueous sodium hydroxide.

10. *Glacial acetic acid.* C.P. grade.

11. *Chloroform.* Reagent grade, freshly distilled.

12. *Sodium bicarbonate.* Reagent grade 8% aqueous solution.

13. *Sodium hydroxide.* Reagent grade pellets. A 1 *N* aqueous solution.

##### *Preparation of the Urine Extract*

A one-hundredth part of a 24-hour collection of urine is added to the glacial acetic acid and the pH is adjusted to 4.6. Then 2 ml. of 2 *M* acetate buffer (pH 4.6), 5.0 ml. of chloroform, and 2500 units of  $\beta$ -glucuronidase are added per 100 ml. of urine. This is incubated at 37°C. for 24 hours and extracted twice with equal volumes of chloroform (or purified methylene dichloride). The solvent extract is washed successively with 1 *N* sodium hydroxide, 8% sodium bicarbonate solution, and then with water to neutrality. The solvent extract is desiccated by placing it over anhydrous sodium sulfate. Following its removal by filtration the solvent extract is brought to dryness under reduced pressure.

*Procedure*

1. To the residue above, which has been transferred to a tube and thoroughly desiccated, is added 0.10 ml. of glacial acetic acid and the residue is dissolved by slight warming if necessary.

2. One milliliter of oxidizing reagent (5 above) is added to the residue.

3. A slow stream of purified air is immediately passed through the oxidizing solution by means of an aeration head having an inlet tube which is immersed directly into the solution and an outlet tube at the tip of the head. The reaction tube must be absolutely sealed by the head to prevent escape of acetaldehyde. The tip of the outlet tube is at once immersed in the sodium bisulfite solution.

4. A flow of air equivalent to 5-cc. volume per minute is sufficient to cause a quantitative transfer of acetaldehyde to the bisulfite solution in 1 hour.

5. To the bisulfite trap solution is added 3.5 ml. of concentrated sulfuric acid solution, mixing well.

6. To the solution which has been cooled in ice water is added 0.02 ml. of the copper and 0.02 ml. of the 4-hydroxydiphenyl reagents.

7. After keeping the tube in an ice bath for 1 hour and stirring at the 30-minute period the excess 4-hydroxydiphenyl is destroyed by heating in a water bath for 1.5 minutes.

8. Optical densities of the solutions are read at 565 m $\mu$ . The amount of acetaldehydogenic substances can be calculated from a calibration curve previously made with pregnanetriol as a standard. Although this method has not been applied for quantitation of blood plasma acetaldehydogenic steroids there is no apparent reason why it should not be successful. The plasma extract can either be prepared by the method of Bongiovanni and Eberlein (1955) or by the method of Silber *et al.* (1958). These methods will not be described here since they are dealt with in the following section.

As was previously mentioned the acetaldehydogenic method is specific for the determination of 17 $\alpha$ ,20-dihydroxy-21-methyl steroids in purified urine extracts. In patients with adrenal hyperplasia most of the acetaldehydogenic steroids are accounted for by those which are isolated on paper chromatograms. The presence of other steroids (11-deoxycorticosterone and 11-deoxycortisol) does not interfere with the recovery of acetaldehyde liberated from pregnanetriol. The urine extracts must be washed as outlined or by following some equivalent procedure. Recovery data were found to vary between 52 to 102% in unwashed extracts compared to 90 to 96% in washed extracts. Methods for the determination of pregnanetriol are discussed in detail in Chapter 4.

### 3. Phenylhydrazine

The reaction given by  $17\alpha,21$ -dihydroxy-20-ketosteroids ( $17\alpha$ -OHCS, group 4, Fig. 1) with sulfuric acid solutions of phenylhydrazine was introduced by Porter and Silber in 1950. The popularity of the Porter-Silber reaction stems from the fact that it is applicable in clinical practice to small samples of both blood and urine. It is believed that the mechanism of the Porter-Silber reaction is based on the formation of a yellow 3,20- or 3,21-bisphenylhydrazone (Silber and Porter, 1957) which has an absorption peak at or close to  $410\text{ m}\mu$  with an  $E_{1\text{cm}}^{1\%}$  of 0.476 for cortisol. The  $\Delta^4$ -3-keto grouping of steroids also reacts with the Porter-Silber reagent but the resulting products have absorption maxima between 340 to  $360\text{ m}\mu$  which do not interfere with the measurement of  $17\alpha$ -hydroxycorticoids (Silber and Porter, 1954). Other substances, however (see below), do interfere and as a result decrease the sensitivity and specificity of the methods.

In the original procedure proposed by Porter and Silber (1950) a benzene-water partition system was used to purify urine extracts. This led to serious losses of steroid unless the water phase was exhaustively extracted with solvent. Recoveries of 10 to 25  $\mu\text{g.}$  of steroid added to 10 ml. of urine or plasma varied between 85 to 91% and 82 to 95%, respectively. Urine blanks were high and turbidity was often seen in extracts of plasma which interfered with the optical density readings.

A new micromethod applicable to 4 ml. of urine or plasma was developed by Silber and Porter (1954) and could be used for clinical studies. It consisted essentially of a four-step procedure wherein the steroids were extracted from urine or plasma before or after  $\beta$ -glucuronidase hydrolysis. The solvent is washed with 0.1 *N* sodium hydroxide and the steroid extracted directly in the sulfuric acid-phenylhydrazine reagent. To obtain lower blanks and greater sensitivity, the following changes from the 1950 method were made: (1) a decrease in the ratio of phenylhydrazine to sulfuric acid to minimize the unstability of cortisol and to obtain more equivalent colors with cortisone; (2) a substitution of ethanol for methanol which sometimes gave pinkish colored products (cf. also Eik-Nes, 1957); and (3) a slower rate of reaction. The specificity of the method was very good when it was applied to normal pooled human urine or plasma and depended to a great extent on the elimination of nonspecific chromogenic substances by the 0.1 *N* sodium hydroxide wash. Thus, it was found that the absorption spectra of steroid-phenylhydrazine reaction solutions of urine and plasma extracts in the range of 350 to  $500\text{ m}\mu$  were almost the same. However, in certain pathological

conditions or after medication a single alkali wash of the urine or blood extracts was insufficient to eliminate nonspecific chromogens.

Marks and Leftin (1954), using the method of Reddy *et al.* (1952) to extract urines, showed that paraldehyde, potassium iodide, and chloral hydrate gave significant absorption values at 410  $m\mu$  which interfered with the specificity of the 1954 Porter-Silber method. Sulfamerazine or sulfadiazine medication resulted in an interfering orange-brown color in the final reaction solution (Lampe-Hintzen and Huis in't Veld, 1955). Although its absorption maximum was not at 410  $m\mu$  the color contributed sufficiently to give rise to erroneous values. The antibiotic triacetyloleandomycin also interferes with the specificity of the Porter-Silber reaction in a similar fashion (Numeroff *et al.*, 1959). On the other hand, Silber and Busch (1955) studied 25 commonly used therapeutic agents and observed little interference in the Porter-Silber reaction. Paraldehyde as well as quinine and its hydrochloride and colchicine were exceptions and gave rise to high absorption values at 410  $m\mu$ . As indicated by Silber and Busch (1955), determinations should be carried out with the substances to be administered if only to ascertain their reacting properties with the Porter-Silber reagents.

In order to overcome some of the idiosyncrasies of the method and because standard steroid still had to be added to correct for losses (20–25%) in the extraction procedure, Silber and Busch (1956) advocated the washing of urine or plasma with carbon tetrachloride and/or petroleum ether prior to extraction with methylene dichloride. The method yielded more satisfactory results since many of the medicaments (or their metabolic products) used in therapy are soluble in one or the other of these solvents. Additional readings were run at 380 and 440  $m\mu$  with blanks (sulfuric acid + extract) and samples to ascertain the presence of a peak absorption at 410  $m\mu$ . Recoveries by this method were good when as little as 0.5  $\mu g.$  of cortisol were added to 2–10 ml. of plasma (average 91.5%). Duplicability of the method was found excellent after checking the amounts of free cortisol in 5- and 10-ml. samples of pooled plasma in triplicate on two occasions.

The greatly increased sensitivity of this method has made it applicable to 2-ml. samples of normal plasma. In the form presented (see below), the method cannot be applied to plasma from jaundiced patients. Also, provision should be made to eliminate paraldehyde and acetone from extracts, especially when analyzing urine or plasma obtained from diabetics. Although Silber and Porter (1957) have shown that steroids other than those possessing the 17 $\alpha$ -hydroxycorticoid side chain (21-aldehydehydres of 11-dehydrocorticosterone and corticosterone, 11 $\beta$ ,21-dihydroxy pregna-4,16-diene-3,20-dione) react with the Porter-Silber rea-

gent they are of no concern since their presence in biological extracts is unlikely. However, Péron (1960) has obtained a substance<sup>1</sup> of adrenal origin which reacts with the Porter-Silber reagent. Although its origin is adrenal, it could conceivably be present in blood and contribute to the absorption values.

The Porter-Silber reaction has also been the basis of procedures initiated by Reddy and co-workers (Reddy *et al.*, 1952, 1955, 1956; Reddy, 1954) for measuring total  $17\alpha$ -OHCS in butanol extracts of plasma or urine. The extreme simplicity and the applicability of these methods for the extraction of total  $17\alpha$ -OHCS should make them invaluable for analyzing a large number of biological samples. However, unless the methods are rigorously standardized, the values obtained with the Porter-Silber reaction can be erroneous. Butanol, in addition to having excellent solvent properties for steroids, extracts many nonspecific Porter-Silber chromogenic material. Commercial butanol, a source itself of nonspecific color (W. R. Smith *et al.*, 1954), must be purified before use. These authors were also able to obtain significant lowering of the blank values by decreasing the concentration of the acid used in the Porter-Silber reagent. Addition of kaolin to urine prior to extraction and acidification overcame emulsions and adsorbed troublesome urinary proteins. However, the blanks obtained here as well as those obtained following other modifications (Reddy, 1954) were of such an order of magnitude that the sensitivity of Porter-Silber reaction was decreased greatly. In addition, variations in color intensities from steroid to steroid was obtained even though the concentrations were the same. For example, tetrahydrocortisol gave only 35% the color of cortisone. To avoid losses by alkali washing of the butanol extracts anhydrous sodium carbonate was added and was found to remove as much nonspecific chromogens as sodium hydroxide (Reddy, 1954). After precipitating the blood protein with zinc sulfate and sodium hydroxide this method was used for the determination of plasma  $17\alpha$ -hydroxycorticoids (Reddy *et al.*, 1956).

The correlation between the amount of substances being measured, and the functional state of the individuals studied, which was supported by the fact that the spectral curves obtained with extracts were similar in shape to those obtained with pure  $17\alpha$ -hydroxycorticoids, was interpreted as strong evidence for the identity of the materials being measured. To increase the validity of the Porter-Silber values obtained with butanol extracts, Hertoghe *et al.* (1955) proposed the application

<sup>1</sup> Note added in proof: Since submission of the manuscript the substance referred to here has been identified as 18-hydroxydeoxycorticosterone or 20,21-dihydroxy-18,20-epoxy-4-pregnen-3-one (F. G. Péron, *Endocrinology*, **69**, 39, 1961).

of the Allen correction equation to minimize the great variability in urinary 17 $\alpha$ -hydroxycorticoid values. A mathematical formula was used which successfully treated all values obtained (370, 410, and 450 m $\mu$ ) regardless of their sign. In 12 duplicate determinations of the same urine the standard deviation amounted to 6% of the mean value whereas a 43% deviation was found with the method of Reddy (1954). Of equal importance was the comparable color intensities obtained with tetrahydrocortisone (THE) and cortisone (E) (THE = 96% E) whereas without the Allen correction THE = 87% E.

Schopman *et al.* (1957) criticized the indiscriminate use of the Allen correction because the Porter-Silber spectral curves of butanol extracts of some urines treated with sodium borohydride showed greater optical density values at 370 than at 380 m $\mu$ . In these cases, results calculated with the Allen correction at one set of wavelengths were substantially different from those found at another. The discrepancies are overcome by reading at 380, 410, and 440 m $\mu$ .

The foregoing directs attention to the necessity of being aware of the limitations of the Allen correction equation. As originally stated by Allen (1950) this correction can be used provided the absorption curves of the contaminating substances closely approximate a straight line and change linearly over the range of wavelengths used for a particular chemical assay. If the curve is irregular, as in the above case, reading at other wavelengths is permissible only if the requirement of linearity is met. The latter should be ascertained especially when innovations are made in routine methods of extraction or estimation. This is done by inspecting a number of curves obtained with the same extract of the material to be assayed but without interference from steroids which are to be measured. The effect of steroids can be eliminated as proposed by Allen (1950) or the interfering substances themselves may be separated from the steroids and their spectral curves obtained (Chang and Slaunwhite, 1955; Diczfalussy, 1955).

Braunsberg (1957) has proposed a method to test the validity of the Allen correction without the isolation of the interfering substance. It can be applied in a routine way to check the linearity of the background absorption. A further simplification and extension of this method was proposed by O'Sullivan (1958) which gave an indication of the confidence that can be placed on the correction process.

To obtain purified urine or plasma extracts containing less chromogenic PS-reacting material other investigators (Nelson *et al.*, 1951; Nelson and Samuels, 1952; Bayliss and Steinbeck, 1953; Bondy and Altrock, 1953; Eik-Nes *et al.*, 1953; Glenn and Nelson, 1953; Vestergaard, 1953; Gemzell, 1955; Kassenar *et al.*, 1955; Eik-Nes, 1957) used more elaborate

purification procedures. The purification of urine extracts by Florisil columns has been successful (Glenn and Nelson, 1953). If necessary precautions are taken in preparing the Florisil column (Eik-Nes *et al.*, 1953) the method has a high degree of reproducibility and is reliable for the measurement of minute amounts of  $17\alpha$ -hydroxycorticoid (Harwood and Mason, 1956). There is also very good agreement between standard cortisol-Porter-Silber chromogen spectra and those obtained with the  $17\alpha$ -OHCS fraction eluted from the column. Biological specificity was obtained since patients with adrenal insufficiency showed no  $17\alpha$ -hydroxycorticoid values and a high correlation of Porter-Silber chromogens was found before and after ACTH administration.

Although the Porter-Silber chromogen in blood plasma from normal subjects seldom exhibits a peak absorbancy at  $410\text{ m}\mu$ , Eik-Nes (1957) showed that a benzene-water partition of the extract prior to its chromatography on Florisil led to a  $17\alpha$ -hydroxycorticoid fraction which peaked at  $410\text{ m}\mu$ . Other methods correcting for nonspecific chromogenicity of extracts and lack of correspondence in color intensities of different  $17\alpha$ -hydroxycorticoids have been proposed. Silber and Porter (1957) by a mathematical treatment of results obtained with the Silber and Busch method (1956) have obtained identical optical density values with tetrahydrocortisol and tetrahydrocortisone. When extracted from water at a level of 5–15 and 5–30  $\mu\text{g}$ , respectively, these substances have been determined with a precision of  $99.0\% \pm 3.65$  (S.E.). Similar results were obtained when the substances were added to urine. Unless provision is made to correct the variations in intensity of the Porter-Silber chromogens of cortisol, cortisone, tetrahydrocortisol, and tetrahydrocortisone as Porter-Silber chromogens all values must be reported in terms of a particular standard equivalent.

There is good agreement between the Porter-Silber methods (Silber and Porter, 1954) and the more elaborate ones of Nelson and Samuels (1952), where Florisil is used to purify extracts. This was shown by Wallace *et al.* (1955) in an intensive study of normal blood and blood obtained from individuals without endocrine diseases. Eik-Nes (1957) has also shown an excellent degree of correlation of the Porter-Silber (Silber and Porter, 1954) method with the slightly modified Nelson and Samuels (1952) method which he proposed (see Section III, B). The specificity of these methods has been checked by paper chromatographic procedure and isotopic dilution methods. In 25 paper chromatographic determinations Migeon *et al.* (1956c) found that  $85\% \pm 19$  (S.E.) of the  $17\alpha$ -hydroxycorticoids measured by the Nelson and Samuels (1952) method was cortisol.

Peterson *et al.* (1957) have found a correlation between the free

plasma cortisol values obtained by the Porter-Silber (Silber and Porter, 1954) method and the isotopic dilution method (Peterson and Wyngaarden, 1956). These authors checked the specificity further by fluorometric assay of the isolated compound (Sweat, 1954) and its reduction product (tetrahydrocortisol) obtained enzymatically and by isolation on Bush partition paper chromatograms (Bush, 1953). The specificity of the procedures has not been checked as rigorously when butanol is used as a solvent.

As was mentioned previously, quantitative paper chromatography of conjugated steroids cannot be carried out with precision (Schneider and Lewbart, 1959). The reliability of the Reddy-type procedures, therefore, can be evaluated only on the basis of a comparison of the values obtained by the Porter-Silber and the Nelson-Samuel procedures.

With an understanding of the limitations discussed above, the Porter-Silber reaction when used in conjunction with various extraction and purification procedures is suitable for routine clinical determinations of free and conjugated  $17\alpha$ -hydroxycorticoids. It cannot be used for the determination of corticosterone and/or its metabolites or  $17\alpha$ -hydroxy,  $17$ -deoxy- $20\alpha$ -, and  $20\beta$ -reduced steroids.

For reasons outlined in the Introduction, the results obtained with a chemical assay should represent the total  $17\alpha$ -hydroxycorticoids. In this manner a truer picture of adrenocortical function can be obtained. The following three procedures are representative methods.

*a. Method of Reddy (1954) and Allen Correction as Proposed  
by Hertoghe et al. (1955)  
Reagents for Urine and Blood*

1. *Butanol*. Reagent grade. The optical density of this solvent should not exceed 0.025 when the color is carried out on 1 ml. of the butanol plus 4 ml. of reagent 5 (Reddy, 1954).

2. *Sodium sulfate*. Reagent grade.

3. *Sodium carbonate*. Reagent grade.

4. *Sulfuric acid*. Concentrated, analytic grade. Reagent: 620 ml. of sulfuric acid added to 380 ml. of glass-distilled water.

5. *Phenylhydrazine hydrochloride*. Recrystallized from ethanol and dried. Reagent: 65 mg. of phenylhydrazine hydrochloride dissolved in 100 ml. of sulfuric acid reagent.

*Reagents for Blood*

1. *Zinc sulfate*. Reagent grade; 10% aqueous solution of zinc sulfate  $7H_2O$ .

2. *Sodium hydroxide*. Reagent grade; 0.1 *N* aqueous solution.



3. 10 ml. of reagent 1 should be equivalent to 10.9–11.2 ml. of reagent 2 using phenolphthalin as the indicator.

#### Procedure

Ten milliliters of blood or 10 ml. of urine; 10 to 20  $\mu$ g. of standard steroid are carried through the procedure used below.

Blood proteins are first precipitated with the zinc sulfate and sodium hydroxide solutions according to the precipitation technique of Somogyi (1930). The proteins in 10 ml. of plasma are precipitated by mixing with 5 ml. each of the zinc sulfate and sodium hydroxide reagents. After centrifugation the clear supernatant is used for the butanol extraction. Protein-free blood and urine samples are treated in the same manner as per protocol.

1. Acidification with sulfuric acid to pH 1.
2. Saturation with sodium sulfate (2.5–3.0 gm. for 10 ml.).
3. Extraction with equal volumes of butanol.
4. Addition of solid sodium carbonate (0.25–0.50 gm.) to the separated butanol phase; solution allowed to stand 5 minutes.
5. Evaporation of the butanol is carried out at a reduced pressure.
6. Addition of the Porter-Silber reagent (4) to all or to aliquots of the extracts.
7. Reading of the tubes at 370, 410, and 450  $m\mu$  set up and containing the substances designated in the following tabulated data (Hertoghe *et al.*, 1955)

Difference between the following solution.	Difference between the optical densities read at		
	370 $m\mu$	410 $m\mu$	450 $m\mu$
Standard + Porter-Silber reagent and standard + $H_2SO_4$ reagent	$a$	$b$	$c$
Butanol + Porter-Silber reagent and butanol + $H_2SO_4$ reagent	$a'$	$b'$	$c'$
Butanol urinary extract + Porter-Silber reagent and butanol urinary extract + $H_2SO_4$ reagent	$A$	$B$	$C$

The following calculations are made

$$1. \text{ For the standard solution } \frac{(b - b') - (a - a') + (c - c')}{2} = S$$

$$2. \text{ For the butanol extract } \frac{(B - b') - (A - a') + (C - c')}{2} = X$$

The concentration of  $17\alpha$ -hydroxycorticoid steroids is then calculated by the formula  $10X/S$ . Since  $S$  values were found proportional to the con-

centration of cortisone (Hertoghe *et al.*, 1955) over a wide range, a direct relationship between  $S$  and  $X$  is justified. When negative values are obtained they are treated according to their sign. If interfering substances are suspected which will yield typical colors with the Porter-Silber reagent, the absorption curve over a wide wavelength range should be carried out after borohydride reduction (Schopman *et al.*, 1958) to ascertain the linearity of the nonsteroidal chromogenic curve.

*b. Procedure of Nelson and Samuels (1952) as Modified  
by Eik-Nes (1957)*

**Urine.** A 30-ml. aliquot of a 24-hour sample is buffered with acetate buffer (pH 4.5), mixed with 10,000 Fishman units of  $\beta$ -glucuronidase, and incubated at 47°C. for 15 hours. After 3 extractions with equal volumes of chloroform, the solvent is brought to dryness under reduced pressure. The residue is partitioned between 3 ml. of benzene and 6 ml. of distilled water 3 times. The combined aqueous layers are then re-extracted 3 times with chloroform (or methylene dichloride). The residue obtained after evaporation of the chloroform is chromatographed on Florisil as outlined below.

**Plasma.** Fresh or frozen plasma is hydrolyzed by the method of Bongiovanni and Eberlein (1958). After mixing 10 ml. of plasma with 2.5 volumes of 95% ethanol the precipitate is removed by centrifugation. The aqueous phase is evaporated to a small volume and reconstituted with distilled water to its original volume. After bringing to pH 4.5 with acetic acid and buffering with 0.1  $M$  acetate buffer (pH 4.5) the mixture is hydrolyzed with  $\beta$ -glucuronidase at 37°C. for 48 hours. Subsequently, this is extracted 3 times with equal volumes of methylene dichloride. The methylene dichloride is evaporated and the resulting residue partitioned between water and benzene as for the urine residue above.

**Chromatography on Florisil.** The dry residue from the benzene-water partitions is dissolved in approximately 40 ml. of chloroform which is poured onto a 10-  $\times$  70-mm. chloroform-washed column of Florisil previously prepared with *dry* washed Florisil (Eik-Nes *et al.*, 1953). Elution is carried out with a further 25-ml. volume of chloroform, 25 ml. of 1% methanol in chloroform, and finally with 45 ml. of 25% methanol in chloroform. The last fraction which is evaporated almost to dryness is transferred quantitatively to a glass-stoppered tube and brought to dryness. After dissolving in 0.2 ml. of absolute ethanol, 0.3 ml. of phenylhydrazine-sulfuric acid reagent is added (16 mg. of recrystallized phenylhydrazine + 10 ml. of a mixture of 190 ml. of glass-distilled water and 310 ml. of concentrated sulfuric acid). The tubes are immersed in water

at 60°C. in the dark for 60 minutes. After cooling, all tubes are read at 370, 390, 410, 430, and 450 m $\mu$ .

Optical density readings are obtained by first zeroizing the spectrophotometer with a chloroform blank carried through the entire procedure. Losses of steroid which occur at different steps in the procedure are assessed by determining the recovery of 1–10  $\mu$ g. of the standard used for the Porter-Silber standard curve when added to urine or plasma. The Allen correction equation is applied to all values. Reading at different wavelengths makes possible the detection of excessive background color (high blanks). Samples which exhibit a greater absorbancy at 390 m $\mu$  than at 410 m $\mu$  are to be questioned (Eik-Nes, 1957).

*c. Method of Silber and Busch (1956)*

This method has been given in its entirety elsewhere (Silber and Busch, 1956; Silber and Porter, 1957) and applied to determinations of free 17 $\alpha$ -hydroxycorticoids in plasma and urine and to conjugated 17 $\alpha$ -hydroxycorticoids in urine. It has not been used extensively for quantitative determinations of conjugated blood 17 $\alpha$ -hydroxycorticoids. To hydrolyze these conjugates in plasma the methods of Bongiovanni and Eberlein can be used as was described above. The procedure involved in the Silber-Busch (1956) method is outlined below.

*Reagents*

1. All solvents used are reagent grade.
2. *Dilute sulfuric acid reagent.* Glass-distilled water (190 ml.) plus 310 ml. of concentrated sulfuric acid.
3. *Blank reagent.* Reagent 2 (100 ml.) is added to 50 ml. of absolute ethanol.
4. *Phenylhydrazine reagent.* Recrystallized phenylhydrazine hydrochloride (65 mg.) is added to 150 ml. of reagent 3.

*Procedure*

Five milliliters of urine or deproteinized plasma hydrolyzed with  $\beta$ -glucuronidase are washed successively with 2 to 3 times their volume of carbon tetrachloride and petroleum ether and the solvent phases discarded. The aqueous phase is next extracted twice with 2.5 times its volume of methylene dichloride. The methylene dichloride extracts are pooled and evaporated to dryness under reduced pressure. This will eliminate any acetone which may be present in the extract. After dissolving the residue in 10 ml. of methylene dichloride it is washed with 1.0 ml. of cold 0.1 *N* sodium hydroxide for 15 seconds. The alkali wash is discarded and the methylene dichloride is equally divided between two tubes. To one, 0.5 ml. of reagent 4 are added and to the other an equal volume of

reagent 3. After shaking vigorously for 15 seconds and removing the solvent phase, color is allowed to develop overnight (16 hours) in the dark at room temperature. Aqueous standards (0.5–5  $\mu\text{g.}$ ) are carried through the same procedure. Because of emulsion formation, the tubes are centrifuged after each extraction step. Emulsions which do not break readily after one centrifugation are frozen in a dry-ice solvent mixture and recentrifuged. The optical density of the samples are read in micro-cuvettes at 380, 410, and 440  $\text{m}\mu$ . After subtracting the readings of the blank from those of the sample at each of these wavelengths the corrected optical densities are calculated by subtracting the sum of the densities obtained at 380 and 440  $\text{m}\mu$  from twice those obtained at 410  $\text{m}\mu$ .

#### 4. Bismuthate Oxidation

The popularity in North America of procedures utilizing the phenylhydrazine sulfuric acid reagents for the estimation of blood and urinary adrenocorticosteroids has paralleled in Europe the method using sodium or potassium bismuthate for urinary adrenal steroid estimation. When added to urine, this latter substance (Brooks and Norymberski, 1952) causes the oxidative scission of the side chain of some 17-hydroxylated  $\text{C}_{21}$  corticosteroids (group 3, a, b, and c, Fig. 1) yielding 17-ketosteroids. The latter are measured colorimetrically by the classic Zimmermann reaction (1935). The difference between 17-ketosteroid values before and after oxidation of urinary adrenocorticosteroids afforded a measure of 17-ketogenic steroids (17-KGS, group 3, a, b, and c, Fig. 1) (Norymberski, 1952). One of the advantages of using this method was that a sample containing as little as 10  $\mu\text{g.}$  of corticosteroid could be measured with ease and simplicity and a second, that a large group of adrenal steroids was measured. Still a third, was the fact that enzymatic methods of hydrolysis were not obligatory for obtaining values for total corticosteroid.

In further studies, Norymberski *et al.* (1953) described the conditions necessary for optimal oxidation of urinary 17-ketogenic steroids. Glucose in low concentration (1%) inhibited the oxidative reaction and the authors stressed the importance of testing urines for the presence of this substance before carrying out the estimation of 17-ketogenic steroids. Addition of excess sodium bismuthate was suggested to overcome this problem. This solution was confirmed experimentally by Jorgensen (1957), Birke *et al.* (1958), and Sobel *et al.* (1958). Jorgensen (1957) used fermentation with yeast to eliminate the glucose. Neither the yeast nor the alcohol produced interfered with the final 17-ketonic steroid assay.

D. C. Smith and Tompsett (1955) reported that sodium bismuthate

and acetic acid in the presence of high concentrations of sodium chloride caused an oxidation of 17-hydroxyandrostanes and 20-hydropregnanes to the corresponding 17- and 20-ketones. This was not substantiated by Norymberski and Stubbs (1956) or by Sobel *et al.* (1958). Further, Norymberski and Stubbs (1956) maintained that the simultaneous presence of urea in the urine prevented the oxidation of hydroxy steroids by bismuthate. Edwards and Kellie (1958) found that urinary chlorides interfered only when their concentration (molarity) was higher than that of the urea present in the urine. Addition of urea, however (100 mg/25 ml.), to the urine excluded any possibility that this could occur. Another urinary constituent which appears to interfere with the bismuthate oxidation in large amounts is dehydroepiandrosterone. In its presence 17-ketosteroids values obtained before and after bismuthate oxidation are erroneous (Jorgensen, 1957). This has important implications if 17-ketogenic steroids are to be determined in the urine of adrenal tumor patients who often excrete large amounts of this substance. Plantin *et al.* (1956) have also found that dehydroepiandrosterone reacts with sodium bismuthate, and they postulated that epoxidation of the  $\Delta^5$ -ethylenic bond occurred, leading to error in the estimation of 17-ketogenic steroids. The error as well as the contribution made by urinary pigments to the colorimetric measurement of native 17-ketogenic steroids (Appleby *et al.*, 1955) is overcome by the introduction of a reductive step prior to oxidation of the steroids to 17-ketosteroids (Appleby *et al.*, 1955; Jorgensen, 1957).

By the combination of reductive and oxidative steps, 17 $\alpha$ ,21-dihydroxy or 21-deoxy-20-ketosteroids are first reduced to the corresponding 20-hydroxy compounds of the type depicted by group 3, b and c (Fig. 1), which are then oxidized by bismuthate to 17-ketosteroids. Introduction of the reductive step prior to oxidation, therefore, not only eliminates errors due to nonspecific material and dehydroepiandrosterone but eliminates from the final measurement all other 17-ketosteroids originally present in the urine. The method has also the advantage of measuring the total 17 $\alpha$ -hydroxycorticosteroids.

In further work, Appleby and Norymberski (1955) suggested a method whereby 17-hydroxy, 20-keto, 21-deoxy steroids could be determined specifically. This depended first on destruction of all adrenal steroids, with the exception of the highly stable 21-deoxysteroids, by boiling in aqueous mineral acid (10% HCl v/v). Consecutive treatment of the urine with sodium bismuthate, sodium borohydride, and sodium bismuthate led theoretically to the selective conversion of 17-hydroxy, 20-ketosteroids to 17-ketosteroids. The 17,20-dihydroxy, 21-deoxy steroids on the other hand are oxidized at the first bismuthate step and thence

reduced to nonoxidizable 17-hydroxy compounds which do not interfere in the Zimmermann reaction. Since these compounds are present in urine in very low concentrations urinary chromogens interfere with their measurement. To account for these, blank determinations have to be carried out and under these conditions a mean of 81% recovery (22 experiments) of  $3\alpha,17\alpha$ -dihydroxy- $5\beta$ -pregnane-11,20-dione was obtained after addition to urine. The isolation of etiocholanolone from extracts of acid-hydrolyzed urine after treatment with oxidizing and reducing reagents in the sequence noted above indicated the specificity of the method. This provided a new analytical method for the group determination of 21-deoxy ketols which have been isolated mostly from pathological urines (Lieberman and Dobriner, 1945; Hirschmann and Hirschmann, 1947; Mason and Strickler, 1947; Miller and Dorfman, 1950; Fukushima *et al.*, 1954; Lieberman *et al.*, 1954). With this method Appleby and Norymberski (1957) indicated that the increase in total  $17\alpha$ -hydroxycorticoids in the fifth to tenth lunar months of pregnancy was due in part (25%) to an increase in the level of 21-deoxy, 20-ketols. The balance of the increase was probably due to the higher levels of 17,20,21-triols and 17,20-diols. Oral administration of as much as 200 mg. of cortisone per day for 2 weeks failed to cause an increase in the level of measured 21-deoxy, 20-ketols.

A method using zinc powder in boiling aqueous acetic acid (Norymberski and Stubbs, 1956) for the differential estimation of  $17\alpha$ -hydroxycorticosteroids (Porter-Silber-reacting material) has been proposed. Because of its complexity and failure to destroy all the chromogenicity derived from cortisone after the reactions, the highly specific Porter-Silber methods (cf. previous section) are still those recommended for measuring  $17\alpha$ -hydroxycorticosteroids.

The complexity of the steroid components in urine extracts does not permit an absolute correlation of 17-ketogenic steroids and total  $17\alpha$ -hydroxycorticoids because of different reaction products which are measured with the Zimmermann reaction. Thus, when only bismuthate is used (for 17-ketogenic steroids) the reaction products measured will consist mainly of 11-ketoetiocholanolone (a) whereas introduction of borohydride reduction prior to bismuthate oxidation (total  $17\alpha$ -hydroxycorticoids) yields mainly 11-hydroxyetiocholanolone (b). Since the Zimmermann color equivalents for (a) = 124 and (b) = 85 (when dehydroepiandrosterone = 100; see Appleby *et al.*, 1955) the lack of correlation becomes self-evident. This picture is further complicated by the drastic hydrolytic procedures used to hydrolyze the steroid glucosiduronides prior to the Zimmermann reaction. These cause the formation of artifacts of hydrolysis from a partial or complete conversion of 11-hydroxy-

etiocholanolone to 3 $\alpha$ -hydroxy-5 $\beta$ -androst-9(11)en-17-one (Birke *et al.*, 1958; Edwards and Kellie, 1958). In addition borohydride reduction of 20-ketones is more rapid than that of 11-ketones so that sufficient time must be allowed for a total reduction of 11-keto groups (Appleby *et al.*, 1955; Plantin, 1958).

The question of the reaction time required for the bismuthate oxidation step has also been the subject of some discussion (Diczfalusy *et al.*, 1955) and has led to procedures wherein it was found necessary to increase the time to obtain complete oxidation of 17-ketogenic steroids (Jorgensen, 1957; Golub *et al.*, 1958; Sobel *et al.*, 1958). In further studies, however, Diczfalusy *et al.* (1958) found that an increased oxidation period was related to and varied with the provenance of the bismuthate reagent. Paradoxically, it was found that the purest reagent (Baker's Analyzed) was the least reactive. Therefore, it is advisable to carry out a time study to establish optimal conditions for complete oxidation of steroids.

Any substance which will affect the Zimmermann color reaction will, of course, interfere with the determination of 17-ketosteroids, 17-hydroxycorticoids, and total 17 $\alpha$ -hydroxycorticoids. Meprobamate, a tranquilizing drug, produces a characteristic reaction with Zimmermann reagents. When ingested, it, or its urinary metabolites, produce a Zimmermann chromogen which has an absorption maximum at 395 m $\mu$ . Fortunately, if the Allen (1950) correction is applied correct values may be obtained (Salvesen and Nissen-Meyer, 1957).

Other materials may also affect the over-all specificity of the bismuthate method. For instance, it is impossible to apply the method in the presence of traces of glycols or glycerols because their oxidation product reacts with Zimmermann reagents. Thus, eluates from Zaffaroni-type paper chromatograms, or those obtained from partition columns using glycols or glycerol as stationary phases must be freed of these interfering substances. It is evident, therefore, that the bismuthate method cannot be used for the analysis of adrenocorticosteroids in solvent extracts of blood or tissue containing lipid. On the other hand, it has been successfully used for the determination of urinary allotetrahydrocortisol isolated on Bush-type paper chromatograms (Bush and Willoughby, 1957).

By taking into account the above problems, the bismuthate or borohydride-bismuthate methods as applied to urine analyses have the qualifications of reliability, reproducibility, precision, and chemical specificity (Edwards *et al.*, 1953; Appleby *et al.*, 1955; Diczfalusy *et al.*, 1955; Borth *et al.*, 1957; Butt *et al.*, 1957; Birke *et al.*, 1958; Gold and Starr, 1959). Recoveries of model 17-ketogenic steroids or total hydroxycorticoids from

urine correlate well with values obtained with other methods (e.g., Porter-Silber) (Butt *et al.*, 1957; Nabarro *et al.*, 1957; Birke *et al.*, 1958; Sobel *et al.*, 1958; Gold and Starr, 1959). The bismuthate reaction may be used for the quantitative determination of pure nonesterified (at the 21 position) 17,21-glycol. If the 21-hydroxyl function is masked with an ester group there is no reaction with bismuthate. However, the same compound after borohydride reduction or 17,20,21-glycerols 21-acetates will be oxidized to 17-ketosteroids. This has led Appleby *et al.* (1955) to indicate that the bismuthate reaction depends upon initial fission of the C-20, 21 bond and the formation of an  $\alpha$ -hydroxy acid.

Edwards and Kellie (1958) in a study of the borohydride-bismuthate methods have approached some of the problems discussed above in a different manner. To eliminate possible interference of glucose and production of acid-hydrolysis artifacts (e.g., 3 $\alpha$ -hydroxy-5 $\beta$ -androst-9(11)-en-17-one) estimation of adrenocorticosteroid is carried out with an ether-ethanol extract of the urine (see Section III, B, 4, b and Edwards *et al.*, 1953). This extract is oxidized in the presence of bismuthate and trichloroacetic acid (TCA) resulting in a simultaneous oxidation and hydrolysis to yield 17-ketosteroids. Since only glucosiduronides are hydrolyzed by the reagents, 17-ketosteroids and 17-ketogenic steroid-glucosiduronide levels can be measured. This method yields essentially the same 17-ketogenic steroid value as when the oxidation step is carried out directly on urine by the usual bismuthate methods. One disadvantage is the introduction of an additional step involving the purification of methylene dichloride extracts with alumina prior to the Zimmermann reaction. Also, many more manipulations must be performed before an extract is obtained which is suitable for chemical assay.

The two following methods incorporate the requirements discussed above.

*a. Method of Appleby et al. (1955) as Modified  
by Birke et al. (1958) for 17-Ketogenic Steroids*

*Reagents* (all analytical reagent grade)

1. *Potassium borohydride.*
2. *Glacial acetic acid.*
3. *Sodium bismuthate.*
4. *Sodium bisulfite.* Reagent, 5% (w/v) aqueous solution.
5. *Concentrated hydrochloric acid.*
6. *Sodium hydroxide pellets.* Reagent, 3 N aqueous solution.
7. *Ethyl ether.* Purified by extraction with 5% aqueous ferrous sulfate and then distilled over sulfuric acid.
8. *Potassium hydroxide.* Reagent, 1.25 N in ethanol containing 40 mg.



of ascorbic acid per 50 ml. of solution. Purer and more stable solutions of potassium hydroxide are obtained by this method (Hamburger, 1952).

9. *m*-Dinitrobenzene. Recrystallized from ethanol until it is pale yellow in color. Reagent, a suitable amount prepared fresh each day by mixing a preparation of 1:2 (v/v) of 2% ethanolic *m*-dinitrobenzene and reagent 8.

#### *Procedure*

To each of two 8-ml. aliquots of a 24-hour sample of urine are added 40 mg. of sodium borohydride. After shaking at room temperature for 1 hour, 8.0 ml. of glacial acetic acid and 3.0 gm. of sodium bismuthate are added, and the mixture is shaken in the dark for 2 hours. After centrifugation, 3 drops of reagent 4 are added to 2.5-ml. aliquots of the clear supernatant which is then diluted with 2.5 ml. of glass-distilled water. Following the addition of 0.5 ml. of concentrated hydrochloric acid the tubes are shaken and immersed in *boiling* water for *exactly* 15 minutes. The rack of tubes is next immersed in cold water and 17-ketosteroids extracted by shaking with 8 ml. of ether. After removal of the aqueous phase by aspiration the ether is washed successively with 2.0 ml. of distilled water and 2.5 ml. of 3 *N* sodium hydroxide. The ether phase is finally purified by shaking with approximately 30 pellets of sodium hydroxide. The purified ether extract is brought to dryness and the residue dissolved in 0.6 ml. of reagent 9. The color is allowed to develop in the dark at room temperature for 60 minutes, then to each tube are added 3.4 ml. of ethanol. Readings are taken in a Beckman spectrophotometer against a reagent blank at 460, 520, and 580  $m\mu$ . The values are calculated by using the Allen (1950) correction equation.

With each series of determinations 2 or more standards are run at a concentration which will give an optical density reading which is consistent with good accuracy. Because of the difference in color intensities produced by different 17-ketosteroids with the Zimmermann reagent, it is imperative that the values be clearly denoted in terms of the standard used.

#### *b. Method of Edwards and Kellie (1958)*

*for 17-Ketogenic Steroids (cf. Birke et al., 1958)*

*Reagents* (all analytical reagent grade)

1. *Trichloroacetic acid (TCA)*. Four per cent (w/v) in 75% aqueous ethanol.

2. *Diluting fluid*. Aqueous sodium sulfate anhydrous 3% (w/v) and aqueous sodium sulfite anhydrous 2% (w/v).

3. *Sodium hydroxide*. Reagent, 1 *N* aqueous solution.

4. *Sodium bismuthate*. Containing less than 5 p.p.m. manganese.

5. *Alumina* ( $\text{Al}_2\text{O}_3$ ).

6. *Potassium hydroxide*. Reagent, 2.5 *N* prepared as in reagent 8 of previous method.

7. *m*-Dinitrobenzene. Reagent, recrystallized as in reagent 9 of previous method. Reagent, 1% (w/v) in ethanol.

8. *Methylene dichloride*. Redistilled.

9. *Ethyl ether*. Purified by extraction with 5% aqueous ferrous sulfate and then distilled from sulfuric acid.

#### *Procedure*

In a 20-ml. aliquot of a pooled 24-hour urine sample 10 gm. of ammonium sulfate are dissolved. The steroid conjugates are extracted 3 times with 10-ml. portions of ether-ethanol (3:1, v/v). The pooled extracts are filtered and brought to dryness under reduced pressure at 40°C. The last traces of water are removed by the addition of small amounts of ethanol. The dry residue is dissolved in ethanol, filtered, and brought to a known volume. Duplicate aliquots containing 20–30  $\mu\text{g}$ . of total 17-ketosteroids (1/250 of a 24-hour urine collection) are next pipetted into glass-stoppered, 40-ml.-capacity test tubes and the alcohol evaporated under a stream of nitrogen at 40°C. The residues are dissolved in 25 ml. of 4% TCA and 2 gm. of sodium bismuthate are added. After shaking in the dark for 2 hours the sodium bismuthate is removed by centrifugation and 20 ml. of the supernatant pipetted into 60 ml. of diluting fluid. The aqueous mixture is extracted once with 10 ml. of methylene dichloride and twice with 5 ml. of the same solvent. The combined methylene dichloride extracts are washed with 5 ml. of reagent 3 and then with water until neutral. The methylene dichloride is evaporated under a stream of  $\text{N}_2$  at 40°C. and the dry residue dissolved in 4 ml. of benzene containing ethanol (5% v/v). Then  $\text{Al}_2\text{O}_3$  (400 mg.) is added and the tubes gently agitated for 2 minutes and centrifuged. Three-millimeter aliquots are evaporated to dryness for the 17-ketosteroid determinations. The dry residues along with standards (20–40  $\mu\text{g}$ .) are dissolved in 0.05 ml. each of ethanol and reagents 6 and 7, respectively. A reagent blank is also prepared and, after mixing, the tubes are placed in the dark for 60 minutes at 25°C. Ethanol (2.50 ml.) is next added and the optical densities of each tube read at 440, 500, 520, and 620  $\text{m}\mu$  in a Beckman DU spectrophotometer. Corrected optical densities are obtained by using the Allen correction equation. The 17-ketogenic steroid value is found by subtracting the 17-ketosteroid values obtained prior to oxidation from those obtained after bismuthate oxidation.

Impurities may cause high readings at 440  $\text{m}\mu$  but are usually corrected by the Allen correction equation. When readings at 500  $\text{m}\mu$  ex-

ceed those obtained at 520 m $\mu$ , the results must be looked upon with skepticism.

### C. FLUOROMETRIC METHODS

#### 1. General

Molecules which are excited by incident radiation sometimes emit light. The light-emitting process is due to a change of the energy states of the molecules going from an unstable to a stable form. In this transition, the excitation energy from the incident light which was absorbed by the molecules is dissipated as light immediately after irradiation ceases and is called fluorescent light. The incident light is usually of a different wavelength than the fluorescent light. Thus, even though fluorescence is intimately related with the absorption spectra of a substance or solution, the fluorescence spectra usually differ in many respects. Under ideal conditions, absorption of incident radiation by solutions or substances of known concentrations follow Beer's and Lambert's laws. On the other hand, under the same conditions, fluorescence is directly proportional to the concentration of the fluorescing material unless the quenching phenomenon interferes. This phenomenon "is believed by many workers to be due to collisions between excited and stable molecules, in which excitation energy is dissipated otherwise than by fluorescence. Such collisions may involve molecules of a foreign substance (giving quenching by impurities) or those of the fluorescent substance itself (self-quenching)" (Braunsberg and Osborn, 1952).

Difficulties arising from quenching as well as from other causes may be experienced in the fluorometric measurement of adrenal steroids in biological extracts. One of these is due to the fact that structurally related steroids have different fluorescent spectra on a qualitative and quantitative scale. For this reason methods and procedures have been devised which will measure one or a small group of adrenal steroids specifically. If a more quantitative picture is desired, it may be obtained by separating all the steroids in the extract by chromatography. Each steroid is then treated individually with fluorescence-inducing reagents. Measurement, however, is limited by the availability of a sufficient number of fluorometric methods which can measure all the steroids.

Mineral acids are usually added to biological extracts or to pure adrenal steroids before fluorescence is induced by incident radiation. When working with extracts, this creates another difficulty which is inherent in the use of fluorometric methods. Because acids react non-specifically with many nonsteroidal substances, the extracts must be

meticulously purified before the addition of the acid reagents. In most cases, the procedures necessary to obtain the degree of purification required are very tedious. For this reason, the fluorometric methods are not usually applicable to routine adrenocorticosteroid quantitation. In addition to the above difficulties, the specificity of fluorometric methods as applied to adrenocorticosteroid quantitation depends on such factors as the concentration of the acid, the temperature, and the time of the reaction (Kalant, 1954, 1958; Goldzieher and Besch, 1958) and must be carefully controlled.

## *2. Sulfuric Acid Fluorescence*

Sweat (1951) reported fluorescence in sulfuric acid solutions of cortisol produced by an adrenal cellular constituent incubated with 11-deoxycortisol. Subsequently Sweat and Farrell (1952) presented a fluorometric method which was outlined in detail later (Sweat, 1954, 1955). Briefly, it consisted of an initial petroleum ether-ethanol partition to remove most of the lipid material from the blood extracts. There next followed a purification step by chromatographing and separating the steroids in the extract on a silica gel column. The fractions known to be eluted with cortisol and corticosterone were then brought to dryness and treated with the reagent (9:1, v/v concentrated sulfuric acid/ethanol). Cortisol and corticosterone could then be quantitatively determined in 5 ml. of human venous adrenal or peripheral blood. The extreme care required for preparation of the silica gel column limits the practical application of this method. The column was found effective for the separation of cortisol from corticosterone fractions but could not delineate cortisol and cortisone or cortisone and corticosterone. Cortisone did not fluoresce and hence did not interfere in the assay. Quenching effects were not observed in this method. Although not applicable to routine clinical assay of adrenocorticosteroids, the method was sufficiently standardized to allow an accurate quantitation of corticosterone and cortisol in normal and in some pathological states (Sweat, 1955).

Peterson (1957) using a slightly modified procedure was able to present rigorous proof of the presence and the identity of corticosterone in extracts of human peripheral plasma run on Bush-type chromatograms by measuring the fluorescence of the eluted corticosterone zones. The sensitivity of the original Sweat procedure for measuring corticosterone was greatly improved by increasing the amount of ethanol used in the final reaction (7:3 v/v concentrated sulfuric acid/ethanol). Under these conditions cortisol gave a galvanometer reading of 20 (corticosterone = 100) whereas the value of the reduced derivatives of cortisol and cortisone were negligible. This showed a fairly high degree of specificity of the

diluted sulfuric acid reagent for corticosterone. Replacement of the initial purification step in the Sweat method (petroleum-ether/80% ethanol) by a Florisil chromatogram (Ely *et al.*, 1958) did not increase the specificity or the sensitivity of the original method.

The difficulties involved in Sweat's method for purifying plasma extracts routinely led Zenker and Bernstein (1958) to propose a purification procedure based on the double-extraction technique of Silber and Busch (1956). By replacing the silica gel column (Sweat, 1954) and the paper chromatogram (Peterson, 1957) with an alkali washing of the chloroform extract of rat plasma the fluorescent method was considerably shortened and simplified. Estradiol-17 $\beta$  and presumably other estrogens which fluoresce in the sulfuric acid-ethanol reagent used (50/50 v/v) were mostly removed by the aqueous sodium hydroxide wash. With this method of purification, fluorescence values of extracts obtained with adrenalectomized animals were of the same order of magnitude as with oophorectomized-adrenalectomized animals. Physiological specificity of the method was reflected in a disappearance of fluorescence in bilaterally adrenalectomized rats. The levels of plasma corticosterone reported for the normal resting rat were high ( $33.3 \pm 1.28/100$  ml.) however, if compared with those obtained by other investigators (Silber *et al.*, 1958; Guillemin *et al.*, 1958).

To obtain more highly purified solvent extracts of plasma and adrenal tissue Silber and co-workers (1958) introduced a petroleum-ether partition prior to extraction of plasma or adrenal tissue (as a homogenate in 13% ethanol) with methylene dichloride. After washing the latter with sodium hydroxide as in Zenker's method the procedure is further simplified by adding the sulfuric acid-water reagent (8:2 v/v) directly to the methylene dichloride. The steroids are completely extracted by the reagent. In this method cortisol gives only 33% of the reading given by corticosterone, and other adrenocorticosteroids which presumably would be extracted from blood plasma in the free form did not fluoresce. In spite of certain specificity limitations this method has practical application in the estimation of free cortisol and corticosterone in man (Silber *et al.*, 1958).

Because the rat produces mainly corticosterone (Bush, 1953; Elliott and Schally, 1955; Vogt, 1955; McKerns *et al.*, 1958; Péron, 1960) the method is especially applicable to the determination of this substance in this animal. Because of its great sensitivity, it can be used to determine the level of corticosterone in the rat adrenal before and after ACTH administration (Silber *et al.*, 1958; Moncloa *et al.*, 1959). It has been used to evaluate suppressive activity of exogenously administered steroids on endogenous ACTH output (Péron and Dorfman, 1958). Other ap-

plications of the method have been directed at determining the relative production of fluorescent- and ultraviolet-absorbing compounds released by incubated rat adrenal glands (Fortier, 1959). This fluorescent method has also been used for the determination of the phase relations of 24-hour periodicities in mouse blood corticosterone (Halberg *et al.*, 1959).

The specificity of the method of Silber *et al.* has been verified by isolating corticosterone, present in rat adrenal glands, on paper chromatograms (Moncloa *et al.*, 1959). The corticosterone quantitated by the fluorometric method of Silber *et al.* (1958) was 126% relative to that found by a standard colorimetric BT assay. When added to plasma the recovery of corticosterone (0.1–2.0  $\mu\text{g./ml.}$ ) in one case amounted to  $100.3 \pm 0.4$  (Guillemin *et al.*, 1958) and 101–107% when added at levels ranging from 0.1 to 0.8  $\mu\text{g.}$  per 0.2–1.0 ml. of plasma (Silber *et al.*, 1958). As with Zenker and Bernstein's method physiological specificity was established. Very low levels of fluorescence (Guillemin *et al.*, 1958; Silber *et al.*, 1958) were obtained in adrenalectomized or hypophysectomized rats. A method has been proposed which eliminates this residual fluorescence (Moncloa *et al.*, 1959) yielding negligible values for rat adrenal corticosterone 24 days after hypophysectomy (Péron *et al.*, 1959). It is simpler than that proposed by Silber *et al.* (1958), as it eliminates reading the extracts in sulfuric acid of different concentrations (see page 253).

So far as is known, the methods discussed above have had limited clinical application. Because they make possible the measurement of only a limited number of steroids in biological material the simplicity of colorimetric methods is still preferred for quantitating adrenocorticosteroids. The fluorometric methods are mostly used for investigation purposes and can be used to supplement the information obtained by the simpler colorimetric methods. In addition, and probably of greater importance, will be their application to the study of effects of steroids at the cellular level and problems related thereto. There is also the possibility that differential fluorometric methods will be developed which will enable the assay of all adrenal steroids which are considered to reflect adrenal cortical function. There have been some suggestions along these lines leading to fluorescent methods which are less sensitive (Goldzieher and Besch, 1958) than those discussed above.

### 3. Phosphoric Acid Fluorescence

Because of the lack of correlation of the estrogen values obtained by bioassay and those by fluorometry (Finkelstein, 1948), Zondek and Finkelstein (1952) indicated that a substance called X fluoresced in phosphoric acid and interfered with the fluorometric assay of estrogens.

They showed that this material was a neutral substance which could be separated with ease from the estrogens by extracting an ether extract of acid-hydrolyzed urine with 1 *N* sodium hydroxide. The ether extract could then be made the subject of further purification and X determined by addition of phosphoric acid which caused it to fluoresce. The substance was isolated from the urine of a case of congenital adrenal hyperplasia and identified as pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol-11-one (pregnanetriolone) (Finkelstein *et al.*, 1953). It is excreted in relatively large amounts only\* in cases of adrenal hyperplasia (Finkelstein, 1959). A closely related steroid (pregnanetriol), which also fluoresces in phosphoric acid, is excreted in large amounts in cases of adrenal hyperplasia (Butler and Marrian, 1937), of adrenal-virilizing tumor (Mason and Kepler, 1945), and, in small quantities in the normal individual (Cox and Marrian, 1953; Finkelstein, 1959). It would appear, therefore, that a fluorometric assay method which would permit the simultaneous determination of both these substances would be valuable in interpreting adrenocortical function.

Because of the lability of pregnanetriol to acid the original method of Zondek and Finkelstein (1952) could not be used because hot acid hydrolysis is involved in the method. This led to the development of an analytical procedure based on an enzyme hydrolysis of urine with  $\beta$ -glucuronidase followed by extraction, purification, etc. (see Chapter 5), which could be used for the simultaneous determination of the aforementioned steroids (Finkelstein and Goldberg, 1957; Finkelstein and Cox, 1957; Cox and Finkelstein, 1957). This method, which is suitable for clinical use, was found to be sufficiently sensitive for the determination of pregnanetriol and pregnanetriolone in a 0.05-*aliquot* of a 24-hour urine sample of individuals suffering from adrenal hyperplasia.

In the normal adult, in very young children and infants, and in certain cases of Cushing's syndrome where the level of pregnanetriolone is extremely low, the sensitivity of the method had to be increased for the detection of 1 to 2  $\mu$ g. of the steroid in a 24-hour urine specimen. In this modification (Finkelstein, 1959), several additional purification steps were introduced to make the elimination of nonsteroidal fluorogenic material almost absolute. Because of considerable pregnanetriol loss, however, the procedure was not found suitable for its determination. Thus, by the use of a highly sensitive, specific, and applicable fluorometric method one can probably differentiate most cases of virilizing syndrome caused by congenital adrenal hyperplasia on the one hand

\* A recent report by R. P. Sherman, R. I. Cox, and A. Gannon has shown that this substance is also excreted in the urine of Stein-Leventhal patients. *Lancet* pp. 260-261, Feb. 4, 1961.

("adreno-cortical heterofunction," see Finkelstein, 1959) and by adrenal tumor on the other. The determination of pregnanetriolone is discussed in detail in Chapter 5.

#### 4. Fluorescence on Paper

Another practical aspect of fluorometric methods is their applicability to direct and quantitative determinations of minute amounts of certain adrenal steroids on paper chromatograms. Ayres *et al.* (1957b) have developed a fluorescence method for the microanalysis of  $\Delta^4$ -3-ketosteroids on paper chromatograms sprayed with an alkaline BT reagent. After the chromatograms are dried the areas containing  $\Delta^4$ -3-ketosteroids are cut out by means of a template which then fits into a paper carrier. This is next positioned so that incident light from a mercury vapor lamp illuminates the steroid spot. The induced fluorescence is measured by means of a suitable photomultiplier and corrected for the fluorescence given by an equal area of the same chromatogram containing no steroid. After extensive purification of urinary aldosterone and cortisol (Ayres *et al.*, 1957b) this method was found applicable to 0.2  $\mu$ g. of  $\Delta^4$ -3-ketosteroids. It is imperative that nearly all impurities be removed from an extract prior to the final chromatography because these may either enhance or quench fluorescence. In an analysis of four  $\Delta^4$ -3-ketosteroids spots (two of them standards), the coefficient of variation of the estimate was found to be between 6 and 10% (Ayres *et al.*, 1957c). The great sensitivity of the method is indicated by a linearity of the fluorescent readings over a concentration range of 0.5 to 4.0  $\mu$ g. of a steroid spot occupying an area less than 2 cm. in diameter.

#### 5. Alkaline Fluorescence

The development of fluorescence in aqueous alkaline solutions has not been possible at the present moment. In fact, the presence of water inhibits or quenches the molecular species which are responsible for alkaline fluorescence. Abelson and Bondy (1955) circumvented this problem by using potassium tertiary butoxide instead of aqueous sodium hydroxide for the determination of  $\Delta^4$ -3-ketosteroids. The temperature, the time at which the reaction is carried out, and the concentration of the reagent are three important factors which affect the results. There is no reaction unless the entire system is kept anhydrous. Because of different peak fluorescence intensities of several steroids (range 54.1–74.8) relative to cortisol ( $\equiv 100$ ) this method cannot be applied to the quantitative determination of a complex mixture of  $\Delta^4$ -3-ketosteroids. Its extreme sensitivity (0.01  $\mu$ g. of cortisone acetate), on the other hand, will make it ideal to confirm the findings obtained by the paper fluorometric



method discussed above or in instances where extremely small amounts of pure  $\Delta^4$ -3-ketosteroids are isolated.

*a. Method of Silber et al. (1958) as Modified by Moncloa et al. (1959) for Tissue and Plasma Corticosterone in the Rat*

*Materials and Reagents* (chemicals are all analytical reagents)

1. *Concentrated sulfuric acid.* Reagent, approximately 30 N, 8 volumes of sulfuric acid are added to 2 volumes of glass-distilled water.

2. *Sodium hydroxide pellets.* Reagent, 0.1 N, 4.0 gm. dissolved in 1 liter of distilled water.

3. *Methylene dichloride.* Prepared as described previously for the BT determination.

4. *Petroleum ether.* Boiling point 30°–60°C.

5. *Corticosterone standard.* Six micrograms per milliliter of absolute ethanol.

6. *Tubes.* Thick-walled glass-stoppered, 10 ml. capacity.

7. *Centrifuge.* Table model.

8. *Spectrofluorometer.* Aminco-Bowman or a comparable instrument.

*Procedure for Corticosterone in the Rat*

*Adrenal tissue.* Rat adrenals cleaned of adherent fat are homogenized with 2.0 ml. of 33% ethanol in an all-glass tissue grinder and made up to a final volume of 5.0 ml. with water (original volume). After centrifugation at high speed (3400 r.p.m.) for 5 minutes the supernatant is frozen until ready for use. Aliquots of 0.5, 0.7, 1.0, and 1.2 ml. respectively, are taken from each original volume and made up to a final volume of 2.0 ml. with 13% ethanol in 10-ml. glass-stoppered tubes. Each sample is washed with 5.0 ml. of petroleum ether which is removed by aspiration. The samples are next extracted with 4.0 ml. of dichloromethane, the aqueous phase removed by aspiration, and then the dichloromethane is washed with 1.0 ml. of ice-cold 0.1 N sodium hydroxide. After careful removal of all the sodium hydroxide phase, 3.0 ml. of reagent 1 are added, the tubes shaken, and the sulfuric acid phase removed and read after 50 minutes in the spectrophotofluorometer. The activation monochromator is set at  $\lambda_{\text{max.}} = 470 \text{ m}\mu$  and the fluorescence monochromator at  $\lambda_{\text{max.}} = 525 \text{ m}\mu$ . The photomultiplier microphotometer sensitivity knob is set at a position which will give a reading of 40 with an uranine sample of known concentration (0.005  $\mu\text{g.}$  per milliliter of water). The slit arrangement is No. 5 on the Aminco-Bowman instrument. With each experimental run, a four-point standard curve is carried out (0.06–0.24  $\mu\text{g.}$ ). In all cases the extraction and washing steps are accomplished by shaking the stoppered tubes by hand for exactly one minute. After each extraction the sample is centrifuged for 5 minutes at 2000 r.p.m.

**Plasma.** Aliquots of 0.6, 1.0, and 1.4 ml. of the plasma are used. After additions of 13% ethanol or water to a final volume of 2.0 ml., the extractions are carried out as above. Since emulsions usually form at the sodium hydroxide washing stage and cannot be completely broken at 2000 r.p.m., 2.0-ml. aliquots of the *clear* dichloromethane extract are transferred to clean glass-stoppered tubes before the addition of sulfuric acid.

**Blank determination.** Because the quenching phenomenon is absent in the analysis of rat plasma or adrenal tissue (Péron and Dorfman, 1959; Moncloa *et al.*, 1959) fluorescence intensity is a linear function of the size plasma or tissue aliquot used. If lines are drawn through the values obtained for the plasma or tissue aliquots and are extrapolated to zero-aliquot concentration they usually intercept the ordinate at points above the origin. Values of  $9.1 \pm 5.8$  ( $\pm$  standard deviation) for 153 plasma determinations and  $9.2 \pm 5.0$  in 238 adrenal tissue determinations were obtained (Moncloa *et al.*, 1959). In each individual determination, therefore, the ordinate plasma and tissue values (blanks), respectively, are subtracted from plasma and tissue readings obtained with a certain known volume of the aliquots. This presumably corrects for "residual fluorescence" (Guillemin *et al.*, 1958) and possibly other nonspecific fluorescing chromogen(s). The corrected fluorometric readings are reported as micrograms of corticosterone from a standard corticosterone curve obtained in the same manner.

#### D. RADIOISOTOPIC METHODS

The application of isotopic techniques in conjunction with reliable chemical assay methods to quantitate steroids has been extremely helpful in resolving problems concerned with rates of metabolism, apparent distribution volumes, and turnover rates of some steroid substances (Brown *et al.*, 1954; Peterson *et al.*, 1955; Migeon *et al.*, 1956c, d, 1957; Ayres *et al.*, 1957a). Such information would have been difficult to obtain with nonradioactive steroids administered in physiological quantities.

The isotopic dilution method proposed by Cope and Black (1958) for the determination of endogenous cortisol output in man deserves special mention because of its relative simplicity. A small quantity of  $C^{14}$ -cortisol of known specific activity (disintegrations per minute per unit weight) is given orally and the urine collected for a 24-hour period. The small amount of administered steroid after absorption becomes part of the cortisol pool. The assumption is made that the administered cortisol is metabolized in the same manner as the endogenous cortisol.

Therefore, all the known and unknown metabolites of the exogenous material will be present in the same proportion as those of the endogenous cortisol. Consequently, a proportionate distribution (or dilution) of radioactivity results in the endogenous cortisol and all its metabolites.

The total  $C^{14}$ -counts (a) in the 24-hour urine pool are next determined. The steroid metabolites are isolated in pure form by paper chromatography and are quantitatively estimated colorimetrically. When the number of counts have been determined, the specific activity of a suitable metabolite (e.g., tetrahydrocortisone) is calculated (b). The daily cortisol production is then estimated by dividing (a) by (b). An absolute value cannot be obtained because not all the products of cortisol metabolism are excreted in the urine during the first day. The values obtained by this method are believed to be in the range of 80 to 100% of the theoretical value (Cope and Black, 1958).

One of the chief advantages of the method, aside from its simplicity, is that the accuracy is not appreciably affected by decreased steroid output as in hypoadrenalism or hypopituitarism. This is in contrast to the standard colorimetric methods in which accuracy is greatly decreased when readings obtained with the extract approximate those obtained with the blank reagents. With the isotopic method, the amount of metabolite which can be isolated (by using larger aliquots of urine) is still sufficient to make its determination accurate.

One limitation of this method is that it cannot be used to detect rapid changes in adrenal activity (e.g., after ACTH administration) since it is based on an almost complete recovery of the material injected and necessitates the collection of 24-hour urine samples. In such cases, standard colorimetric methods or the isotopic method proposed by Peterson and Wyngaarden (1956) must be used.

Still simpler methods incorporating the principles of isotopic dilution have been proposed for the determination of individual adrenal steroids in blood and urine extracts (Peterson, 1957; Ayres *et al.*, 1957a).

An exact amount of standard steroid of known specific activity, and chemically identical with the steroid to be isolated, is added to the extracts. After preliminary purification(s) of the extract with sodium hydroxide (Peterson, 1957) or on silica gel chromatograms (Ayres *et al.*, 1957a) the steroid is isolated on paper chromatograms in a pure form and the specific activity and the amount present accurately determined. The substance is considered pure when the specific activity remains constant after several purifications yielding the free substance or a derivative. The quantitative recovery of the material sought is not essential since its concentration in the original extract is calculated from the specific

activity data. Thus, the specific activity of the original pure steroid added when divided by that which is obtained for the substance eluted from the final paper chromatogram gives a measure of the steroid present in the original extract.

Two prerequisites limit the application of this method. One is the availability of specific and sensitive methods for quantitating minute amounts of the steroids isolated, and the other is the availability of the radioactive steroid to be added to the original extract. The first is partially fulfilled by fluorometric methods (see previous sections) which can be applied to some but not to all adrenocorticosteroids. The second is more difficult to satisfy since at present only a limited number of adrenocorticosteroids with sufficiently high specific activities are commercially available. For these reasons, isotopic methods such as these are still limited for use at the practical and clinical level where large numbers of determinations must be carried out. No doubt, in the near future a greater selection of radioactive steroids will become available in sufficient quantities and numbers to make their use commonplace in routine analysis.

#### E. OTHER METHODS

In addition to the methods and procedures discussed in the foregoing sections others have been proposed for estimating adrenocortical hormones. For instance, Gornall and MacDonald (1953), instead of using the sulfuric acid-phenylhydrazine hydrochloride reagent as in the Porter-Silber method (Porter and Silber, 1950), employed 2,4-dinitrophenylhydrazine in an alkaline medium to form the colored products of the steroids. The steroid phenylhydrazones which were formed were then measured colorimetrically. Unfortunately, as indicated by Gornall and MacDonald (1953), the method was laborious. Two objections which contraindicate its use are: (1) The blank color fades on standing and (2) a wide variation in optical densities obtained with different steroids limits the quantitation of complex mixtures of adrenocorticosteroids found in biological extracts.

Other methods of determining adrenocorticosteroids which are exemplified by the polarographic method of Morris and Williams (1953) or by oscillographic polarography (Morávek, 1959) can be used. However, their application to routine analysis of adrenal steroids is not practical. In fact, at present the technical difficulties in applying these methods to quantitating steroids are such that they must be carried out by highly competent workers using special instruments. They are used, therefore, mostly at the research rather than at the routine level.

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## Chapter 7

# Assay of Aldosterone and Metabolites

S. A. S. TAIT AND J. F. TAIT

I. Biological Assay	266
A. Introduction	266
B. Rat Assay	267
C. Dog Assay	269
D. General Discussion	270
II. Physicochemical Assay	276
A. Quantitative Methods	276
B. Methods of Identification	286
III. Methods of Purification	293
A. Initial Purification	293
B. Paper and Column Partition Chromatography	296
C. Other Methods	299
IV. Metabolism of Aldosterone in Man	300
A. Pattern of Urinary Metabolites in Normal Subjects	300
B. Abnormal Metabolism	304
V. Urinary Assay Methods	305
A. Hydrolysis of 3-Oxo Conjugate	305
B. Measurement of Aldosterone Released from 3-Oxo Conjugate	307
C. Glucuronide Metabolites of Aldosterone	314
D. Assay of Free Aldosterone in Urine	316
VI. Indirect Secretion Rate Estimates in Man	318
A. Assumptions	318
B. Methods	321
VII. Assay in Peripheral Blood	323
A. Direct Methods	324
B. Indirect Methods	325
C. Plasma Protein Binding of Aldosterone	327
VIII. Assays of the Adrenal Gland	327
A. Adrenal Gland and Incubate Analysis	327
B. Adrenal Venous Blood	328
References	331

Because of the recent intense interest in the raised amounts of aldosterone in various clinical conditions and also in the mode of control of the hormone, there are now a large number of methods of assaying aldosterone. These are so numerous that it is impossible for one group of investigators to have had direct experience of all the methods. Therefore, while the authors intend to describe representative examples of

all types of assay which are of practical importance, when there is a choice of method, they have preferred to present details of the ones with which they have had direct experience. It is not implied that these are necessarily the best available. In any case the choice of method will depend to a considerable extent on the equipment available to the operators.

## 1. Biological Assay

### A. INTRODUCTION

The development of a sensitive electrolyte bioassay for the measurement of the effects of adrenocortical steroids together with that of paper chromatographic systems by Bush (1952) and Zaffaroni and Burton (1951) for their separation were the two major advances in methodology which enabled the successful isolation and identification of aldosterone in adrenal gland extracts, adrenal vein blood, and human urine. Much of the early information concerning the production and excretion of this hormone, both in physiological and pathological conditions, was obtained by means of biological assay. Following the isolation of aldosterone, methods based on the physicochemical properties of this steroid were developed for the routine assay in urine and adrenal vein blood. However, since these have only been developed in the last few years, bioassay is still being used by several groups for estimations of this compound in biological materials. Latterly bioassay has also been used for the detection of possible sodium-excreting compounds and for the investigation of aldosterone antagonists which are now being synthesized. A consideration of the bioassay methods for these purposes would therefore seem to be relevant at this time.

During the early studies on the biological effects of adrenocortical extracts only indirect methods using growth and survival of adrenalectomized rats (Cartland and Kuizenga, 1936; Grollman, 1941) or the muscle fatigue test of Everse de Fremery (Reichstein and Shoppee, 1943) were available for the assessment of activity with respect to electrolyte metabolism. These methods were, of necessity, of long duration and required large amounts of material. The more direct methods which measured the alteration of the levels of sodium and potassium in plasma or urine of intact or adrenalectomized dogs (Hartman and Spoor, 1940; Harrop *et al.*, 1936; Thorn and Engel, 1938) or rats (Wirz, 1950) were subject to wide variations arising from external factors such as diet or temperature. Large amounts of material were still required but estima-



tions of urinary sodium had the advantage of ease of technique as well as sensitivity.

In 1947 Dorfman *et al.* suggested that the excretion of radioactive sodium by adrenalectomized rats could be used as the basis for a sensitive and simple method for the assay of the sodium-retaining effects of adrenocortical steroids since as little as 1  $\mu$ g. deoxycorticosterone could produce significant sodium retention compared with oil-injected controls. A similar procedure based on the increased excretion of radioactive potassium (Dorfman, 1949) promised equal sensitivity. As a result of these findings, several groups were stimulated to develop quantitative procedures suitable for the assay of small amounts of material. The main technical difficulties were considered to be the ensuring of complete voiding of the bladder before injection of the test materials and the subsequent quantitative collection of the urine. Failure to accomplish the former could lead to the excretion of sodium unaffected by the action of the steroid, thus reducing the sensitivity of the method, and incomplete collection of urine could lead to increased variation. Most of the methods evolved used a high sodium or water load and/or ligature of the urethra to reduce these effects.

## B. RAT ASSAY

### 1. *Estimation of Radioactive Sodium and/or Potassium*

(a) Dorfman *et al.* (1947) employed adrenalectomized rats weighing 100–170 gm. The material to be tested was injected subcutaneously in 0.25 ml. corn oil followed 1 hour later by the injection of 1 or 2 ml. of an aqueous solution of sodium chloride (35  $\mu$ g. per gram body weight) containing a tracer dose of  $\text{Na}^{24}$  after which urine was collected for 6 hours. The urine samples were dried and assayed for radioactivity and the results expressed as radioactive sodium excreted as a percentage of the injected dose. Under these conditions deoxycorticosterone as the free alcohol or acetate produced sodium retention compared with the oil-injected controls whereas cortisone and cortisol at levels of 50  $\mu$ g. or more produced sodium excretion. The method has been used for the assay of sodium-retaining activity of a number of steroids but no dose response curve has been published.

(b) Singer and Venning (1953), using a slight modification of this procedure, involving the ligature of the urethra at the same time as the injection of the test material and tracer dose of  $\text{Na}^{24}$ , obtained a linear response against log dose of 1–20  $\mu$ g. deoxycorticosterone acetate. There was considerable day-to-day variation in the sensitivity of the animals

which could be overcome in part by expressing the results as a percentage reduction of the mean control value.

(c) The method of Simpson and Tait (1952) was an extension of the Dorfman procedure whereby  $\text{Na}^{24}$  and  $\text{K}^{42}$  were injected simultaneously in a solution containing a very low sodium and comparatively high potassium load (27 and 381  $\mu\text{g.}$ , respectively). The time of urine collection was shortened to 2 hours after the injection of the isotopes, the compounds to be tested being injected in 0.1 ml. of 10 or 20% aqueous ethanol 1 hour earlier. It was hoped by this procedure to approach a true balance experiment which would also permit the measurement of the effects of such steroids as cortisol and cortisone whose action was of short duration only (Wirz 1951; Kagawa and Van Arman, 1957). The assay, which employed immature adrenalectomized rats (40–50 gm.), was sensitive to 0.83  $\mu\text{g.}$  deoxycorticosterone acetate and 0.4  $\mu\text{g.}$  of the free alcohol and gave a linear response to log dose deoxycorticosterone acetate over a range of 0.83 to 4.23  $\mu\text{g.}$  Cortisone and cortisol, in contrast to the findings of Dorfman *et al.* (1947) in their assay, caused a lowering of the  $\text{Na}^{24}/\text{K}^{42}$  urinary ratio giving a linear regression against log dose and a potency ratio, using deoxycorticosterone acetate as standard, similar to that found in growth, survival, and Everse de Fremery muscle tests.

## 2. Estimation of Inert Sodium and/or Potassium

Although the ease and speed of the measurement of the radioactive isotopes had much to commend it, the limited availability of the short-lived isotopes to some laboratories made the measurement of inert sodium and potassium more convenient. In most cases it was found advisable to inject a high sodium and/or water load.

(a) The method of Marcus *et al.* (1952) increased the load to 5 ml. of 0.9% sodium chloride by intraperitoneal injection which was given at the same time as the subcutaneous injection of the test material in oil. A reduction in the time of urine collection to 4 hours was found to increase the accuracy of the measurement. Urine was collected from pairs of rats and the sodium and potassium estimated by flame photometry. A linear response was obtained when the urinary sodium excreted was plotted against the logarithm of the dose over a range of 2.4 to 60  $\mu\text{g.}$  deoxycorticosterone acetate. No notable increase in accuracy was obtained when possible combinations of the data were analyzed for the construction of a multivariate assay curve.

(b) Farrell and Richards (1953) used essentially the same procedure except that no sodium load was given and urine was collected from

single rats over a 6-hour period. The response was expressed as the lowering of the Na/K urinary ratio.

(c) Deming and Luetscher (1950), also using flame photometry for the measurement of sodium and potassium, found that rigorous control of sodium intake and the substitution of a high water for a high sodium load led to increased sensitivity and decreased variation in their assay. The method, modified by Johnson (1954), used a cross-over test as suggested by Spencer (1950) which eliminated the variation in response from rat to rat. The results were expressed either as log K/Na urinary ratio or as  $\sqrt{\text{Na}}$  excretion, both in terms of the corresponding values obtained after solvent control and standard injections. The log dose response was linear from 2 to 10  $\mu\text{g}$ . deoxycorticosterone acetate.

(d) In order to increase the sensitivity and precision of the assay, Kagawa *et al.* (1952) combined a high sodium load (8.29 mg. as 2.5 ml. of 0.85% sodium chloride solution) with ligature of the urethras. The material to be assayed was given as a divided dose at 0 and 3 hours to ensure continuing activity during the period of urine collection. This was limited to 2 hours starting 1 hour after the second injection of the test material. The results were expressed as sodium appearing in the urine as a percentage of the administered load. A colorimetric procedure was used for the determination. A linear log dose response was obtained between 1 to 12  $\mu\text{g}$ . deoxycorticosterone acetate.

### C. DOG ASSAY

The use of the adrenalectomized dog permitted Liddle *et al.* (1955) to develop an assay procedure which could also give some additional information on the acute physiological effects of the steroids tested. The material to be tested was injected intravenously thus avoiding variations in activity which could arise from different rates of absorption from the site of injection. The urine was collected by means of a retention catheter and hourly collections were analyzed for sodium and potassium by flame photometry. The cross-over design was employed to reduce variation caused by differences in sensitivity from dog to dog and the precision was also increased by expressing the results in terms of the "aldosteroid index." This represented sodium and potassium excretion during the second and third hours after injection expressed in terms of excretion in the 1st hour, when no consistent effect could be observed on either of these elements. No additional information was furnished by the inclusion of results obtained in hours 4 and 5. The log dose response curve was linear over a range of 25 to 800  $\mu\text{g}$ . deoxy-

corticosterone acetate, 0.187 to 2  $\mu$ g. aldosterone, and 1- to 32-hour equivalents of urinary extract, all of which showed no significant departure from parallelism.

#### D. GENERAL DISCUSSION

The salient features of the methods described above are summarized in Table I. Two major differences are apparent; the first lies in the response to "glucocorticoids" such as cortisone and cortisol and the second in the difference in the potency ratio of aldosterone to deoxycorticosterone. The explanation for these differences probably cannot be attributed to a single cause since the action of adrenocortical steroids on mineral metabolism is of a very complex nature and includes the regulation of body compartment concentrations as well as effects on glomerular filtration rate and proximal and distal tubular reabsorption.

##### 1. *Effect of Glucocorticoids*

It seems that either a high water or sodium load favors sodium excretion rather than retention under the influence of cortisol and cortisone. Thus Simpson and Tait (1952) found that under load conditions similar to those used by Dorfman *et al.* (1947) cortisone acetate produced sodium excretion but had no effect on the  $\text{Na}^{24}/\text{K}^{42}$  urinary ratio, a finding similar to that of Johnson (1954) on the effects of 17-hydroxy-steroids on  $\sqrt{\text{Na}}$  excretion and the urinary K/Na ratio. The explanation for this may lie in the action of these steroids on glomerular filtration rate which is superimposed on their tubular reabsorption effects as has been suggested by Streeten *et al.* (1955). This, however, may not be the only factor involved. Wirz (1951) and Kagawa and Van Arman (1957) have shown that the effect of steroids such as cortisone and cortisol on electrolyte metabolism are of short duration only and that the period of sodium retention is followed by marked sodium diuresis similar to that found by Simpson and Tait (1950) for deoxycorticosterone. The net effect may be one of sodium loss, thus masking the period of retention unless a suitable time interval is used for the measurement.

##### 2. *Potency Ratio*

The very high potency ratio of aldosterone to deoxycorticosterone in the "ratio method" of Simpson and Tait (1952) is not yet fully understood. The use of a short time interval for the collection of urine is probably an important factor. The low potency ratio found by Desaulles *et al.* (1953) using a modification of the Kagawa test where urine is also collected for 2 hours only, could be explained in part by the administra-

TABLE I  
CHARACTERISTICS OF THE BIOASSAY METHODS COMMONLY USED FOR THE ESTIMATION OF ALDOSTERONE IN BIOLOGICAL FLUIDS  
AND TISSUE EXTRACTS

Authors	Test animal	Urinary function analyzed against log dose	Sensitivity, minimum detectable dose ( $\mu\text{g./animal}$ )	Potency ratio aldosterone/DOC	Index of precision, $\lambda = s/b$	Index of sensitivity, <sup>a</sup> $\mu\text{g.}$ aldosterone/test	Effect of 11,17-oxysteroids on assay
Farrell <i>et al.</i> , 1954 I	adx rats 150-200 gm.	Lowering Na/K urinary ratio	Aldosterone, 0.03	48	0.46	2.2	—
Johnson 1954 II	adx rats 150-160 gm.	Log K/Na excretion or Na excretion both as function of controls <sup>b</sup>	DOCA, 2.5	36	0.27 0.48	2.0 6.2	No effect on log K/Na ratio; sodium excretion with >50 $\mu\text{g.}$ cortisone or cortisol
Kawaga <i>et al.</i> , 1952 III	adx rats 150-185 gm.	Na excretion as % injected load	DOCA, 2	25 <sup>c</sup> 30 <sup>d</sup>	0.26 —	2.0 <sup>c</sup> 1.7 <sup>d</sup>	12 $\mu\text{g.}$ cortisol no effect; increased sodium excretion 48-500 $\mu\text{g.}$ cortisol/
Marcus <i>et al.</i> , 1952 IV	adx rats 150-170 gm.	Na excretion	Aldosterone, 0.12 <sup>e</sup> DOCA, 2.4	60 <sup>e</sup> —	0.18 <sup>e</sup> 0.31	2.9 <sup>e</sup> —	10 $\mu\text{g.}$ cortisol produced sodium retention; >50 $\mu\text{g.}$ cortisol and cortisone produced increased sodium excretion
Simpson and Taft 1952 V	adx rats 40-50 gm.	Lowering Na <sup>24</sup> K <sup>42</sup> urinary ratio	Aldosterone, 0.01	50	0.15	2.2 <sup>g</sup>	Cortisol and cortisone produced lowering of Na <sup>24</sup> /K <sup>42</sup> ratio but 2000 times less active than aldosterone
Venning <i>et al.</i> , 1956 VI	adx rats 160-170 gm.	No excretion as % mean control	DOCA, 2	25	0.24	1.80	1-10 $\mu\text{g.}$ no effect; sodium excretion >25 $\mu\text{g.}$ cortisone and cortisol
Liddle <i>et al.</i> , 1955 VII	adx q dogs 15-20 kg.	"Aldosteroid index" as function of (K response)-Na response <sup>g</sup>	Aldosterone, 0.9	30	0.24	19.9	Increased sodium excretion; no effect on aldosteroid index below 1 mg. cortisone

<sup>a</sup> Index of sensitivity represents the minimum amount of aldosterone required for a 4-point balanced assay to give fiducial limits of 66-150% at  $P$  0.95 calculated on the basis of the sensitivity and  $\lambda$  for each assay.

<sup>b</sup> Cross-over test used. The response ( $Y$ ) =  $U$  minus  $[(C + S)/2]$  where  $U$  = averaged log K/Na for each rat when the test substance was given and  $C + S$  the corresponding volumes for standard and solvent controls.

<sup>c</sup> Desaulles *et al.*, 1953.

<sup>d</sup> Gaunt *et al.*, 1955.

<sup>e</sup> Rosemberg *et al.*, 1960. Each observation obtained from 2 rats. Lowest effective dose 0.14  $\mu\text{g.}$  aldosterone and 8.4  $\mu\text{g.}$  DOC per rat.

<sup>f</sup> Rosemberg, personal communication.

<sup>g</sup> "Aldosteroid index" = 2.3 (K response). "Na response" represents potassium excretion in hours 2 + 3 - K excretion hour 1 after injection. "Na response" = sodium excretion in hours 2 + 3 - 50 + 0.4 (sodium excretion in hour 1); 50 + 0.4 = weight factor for sodium response.

tion of the test materials as a divided dose 1 and 4 hours before the start of the collection. Then, if the action of aldosterone is of short duration only, the first injection may have little or no effect during the period of urine collection. In addition, the low sodium and relatively high potassium load used in the "ratio method" could be a contributing factor. Whatever the reason for the high activity of aldosterone in this test may be, it is quite clear that the injection of the radioactive isotopes is not required for this response. Mattox *et al.* (1953a, b) using essentially the same method but estimating sodium and potassium by flame photometry, also obtained a potency ratio of aldosterone to deoxycorticosterone acetate of approximately 100:1.

### 3. Choice of Response Parameter

The increased availability of accurate and reliable flame photometers has led to a critical examination of the radioactive procedures for the measurement of sodium and potassium. Venning *et al.* (1955) reported very good agreement for the log dose response curves of the inert and radioactive methods and the injection of a tracer dose of  $\text{Na}^{24}$  was therefore omitted in their later work (see Table I, Method VI). Similarly R. M. Jones *et al.* (1957) carried out an analysis of variance for the two procedures and found no significant difference in the regression coefficients nor in the position of the controls to the regression lines. There was a slight superiority in the inert method which could only be attributed to the smaller error variance, probably the higher error of the radioactive method being due to the variation from animal to animal in the distribution of the isotopes after injection. Essentially the same findings have been reported by Llaurodo (1956). Mattox *et al.* (1953a, b) found very little difference in the potency ratio of aldosterone to deoxycorticosterone acetate whether the inert or radioactive ratio was measured. In their later work they preferred the radioactive method but no reason was given for this and there appears to be little justification for the development of more accurate but also more complex procedures such as  $\gamma$ -ray scintillation spectrometry (Orvis and Albert, 1955) for the estimation of the  $\text{Na}^{24}/\text{K}^{42}$  ratio for this purpose.

The precision which can be obtained using these assays is dependent on a number of factors. Most workers agree that the postoperative environmental conditions play an important part and strict temperature and humidity control as well as a constant diet are essential if consistent results are to be obtained. Variation from animal to animal can also be reduced by choosing a suitable response metameter. Thus a bivariate analysis of variance or "analysis of dispersion" by R. M. Jones *et al.* (1957) of  $y_1 = \log \text{Na}^{24}$  and  $y_2 = \log \text{K}^{42}$  showed that  $\text{Na}^{24}$  alone yielded

much less information than  $\text{Na}^{24}$  and  $\text{K}^{42}$  together. No extra information was provided by considering  $\text{Na}^{24}$  and  $\text{K}^{42}$  separately in addition to their ratio. It was also found that  $\log \text{Na}^{24}/\text{K}^{42}$  was a more suitable metameter than  $\text{Na}^{24}/\text{K}^{42}$ . Johnson (1954) and Singer and Stack-Dunne (1955) likewise found the log of the ratio to be more satisfactory in that it gave a standard deviation which did not vary systematically with the mean. Similarly there was an increase in precision when the effect on potassium as well as on sodium was included in the response in the inert measurements, e.g.,  $\lambda = 0.27$  for the ratio compared with 0.48 for  $\sqrt{\text{Na}}$  (Johnson, 1954) and  $\lambda = 0.24$  for "aldosteroid index" (see footnote *g*, Table I) compared with 0.37 and 0.48 for potassium and sodium, respectively (Liddle *et al.*, 1955).

#### 4. Index of Precision and Sensitivity

It can be seen from Table I that most of the bioassays have a very similar index of precision but show considerable differences in their sensitivity to aldosterone (amount necessary per animal). In order to provide a valid basis for comparison with physicochemical procedures, a factor based on the index of precision and sensitivity has been calculated for each of the assays listed (see Table I "index of sensitivity"). This states the minimum total amount (given to all the animals) of aldosterone required for a 4-point balanced assay to give fiducial limits of 66–150% at  $P=0.95$  (standard error about 20%) an error similar to that of most physicochemical estimations. With the exception of the dog assay (Liddle *et al.*, 1955) the over-all sensitivity of the biological methods is of the same order as that of the majority of the physicochemical procedures (see Tables III and IV, Section II), and only the methods using labeled reagent techniques and the most sensitive versions of the soda fluorometer method have greater sensitivity than the "ratio method" (Table I, assay V "index of sensitivity" 0.4  $\mu\text{g.}$ ).

#### 5. Salt-Excreting Effects

Although there is little to choose between the physicochemical (except those employing labeled reagents) and biological methods on the basis of sensitivity and accuracy, the former have certain advantages which have become apparent during the last few years. Thus as bioassay was initially carried out on crude urinary extracts most workers reported a lack of correlation between the biological effect and the amount of urinary extract tested (Singer and Venning, 1953; Deming and Luetscher, 1950; Genest, 1956), and some further purification of the extract was found to be essential. In some instances a single chromatogram on paper (Lieberman and Luetscher, 1960) or partition column (Ayres *et al.*,

1957c) was sufficient to remove impurities and to give good agreement between the physicochemical and biological methods. However, in other cases more extensive chromatography was found to be necessary; thus Venning *et al.* (1956) reported that a single chromatography in the Bush B5 system (Bush, 1952) was insufficient to remove all the interfering substances. Methods based on sodium excretion alone proved to be particularly susceptible to the presence of interfering compounds. Thus in a careful comparison between bioassay, ultra-violet absorption, and blue tetrazolium reaction, Dyrenfurth and Venning (1959) found that even in normal pregnancy chromatography in two (chloroform-formamide and Bush C) or three (Zaffaroni propylene glycol-toluene, Bush C and Eberlein and Bongiovanni E<sub>2</sub>B) paper systems was required for agreement between the biological and chemical estimations. Moreover, in some pathological conditions even this extensive chromatography did not remove all the compounds interfering with the biological estimation. Under such conditions there is clearly no advantage in using bioassay for the estimation of aldosterone particularly as the formation of the acetyl derivative, which has proved a useful tool in the separation of aldosterone from other 3-oxo-4-enes, cannot be applied in this instance. It has been recognized since the early work that the diacetate of aldosterone had very little biological activity (Grundy *et al.*, 1952). Mattox *et al.* (1953a, b) reported that aldosterone diacetate was about equipotent with deoxycorticosterone acetate in their assay, i.e., some 100 times less active than the free compound or the 21-monoacetate. Acetylation on the 18 position also substantially reduced biological activity to some 15 times that of deoxycorticosterone (Mattox, 1955). Therefore, purification through the diacetate would require preliminary hydrolysis before biological assay. However, this procedure has been developed into a reliable method by Farrell *et al.* (1954).

From these considerations of the bioassay procedures it is apparent that methods using the combined effects on sodium and potassium excretion as the response are more suitable for the estimation of aldosterone in biological extracts since they are less susceptible to interference from the presence of compounds such as cortisone and cortisol (see Table I). However, if information is required concerning the presence of possible sodium-excreting compounds, then assays using a high water and/or sodium load and the measurement of sodium and potassium excretion individually would be more applicable. Such methods have been used by Johnson (1954), Axelrad *et al.* (1954), Desaulles (1959), Uete and Venning (1960), and Rosenberg *et al.* (1960) to demonstrate the natriuretic effects of synthetic and naturally occurring compounds. There are some disadvantages in the use of the rat as an assay animal for this purpose



since, as pointed out by Uete and Venning (1960), it is technically difficult in small animals to separate the effects on tubular reabsorption from those on glomerular filtration, and Kagawa *et al.* (1957) have, therefore, preferred to use the urinary Na/K ratio to sodium excretion for measuring the blocking effects of the spiro lactones on the salt retention produced by aldosterone and deoxycorticosterone. In addition, as discussed by Gaunt (1959), there are a number of chemical substances which act as sodium and water diuretics in the rat but have little or no activity in dogs or human subjects. Thus 3 $\beta$ , 16 $\alpha$ -dihydroxypregnan-20-one which is natriuretic in the rat (Desaulles, 1959) appears to be devoid of action on sodium or water excretion in man (Coppage and Liddle, 1960). The use of dogs as an assay animal may therefore be preferable for, although susceptible to alterations in glomerular filtration (Garrod *et al.*, 1955), studies on renal function can be carried out and the effects on glomerular filtration and on sodium and water exchange more clearly defined when using methods such as that of Liddle *et al.* (1955).

#### 6. Biological Assay in Blood

Although it is now a relatively simple matter to measure the secretion rate of aldosterone in man (see Section VI, B), the methods are not yet applicable to experimental animals. Bioassay is therefore still being used for the estimation of aldosterone in adrenal vein blood to assess the production rate of this hormone by the adrenal gland (Das Gupta and Giroud, 1959; Ganong *et al.*, 1959). In these cases a single chromatographic separation has been found sufficient to give a valid result. However, at the present time no bioassay has sufficient sensitivity for the estimation of this hormone in reasonably small volumes of peripheral blood. Coghlan *et al.* (1960) have made ingenious use of the biological effects of aldosterone to estimate the levels of this hormone in the blood of sheep by comparing alterations in the Na/K salivary ratio in animals with parotid fistulas with the lowering produced by intravenous infusion of different doses of *dl*-aldosterone under standardized conditions. The observed changes in the Na/K salivary ratio could then be correlated with alterations in the secretion rate of aldosterone under different physiological conditions. The validity of these measurements has been confirmed by concomitant direct estimations of aldosterone in adrenal vein blood (Denton, 1961). Unfortunately this technique is not easily applicable to other experimental animals or to man where changes in the concentration of salivary sodium and potassium are small and the response to aldosterone may be obscured by fluctuations in the rate of salivary flow.

## II. Physicochemical Assay

The isolation (Simpson *et al.*, 1954a) and elucidation of the structure of aldosterone (Simpson *et al.*, 1954b), by providing information on many physical and chemical properties of the free compound and its derivatives, stimulated the development of methods which did not involve bioassay.

The following reactions suitable for micro work have been used for the measurement and identification of the hormone.

### A. QUANTITATIVE METHODS

#### 1. Ultraviolet Absorption

The ultraviolet absorption spectrum of aldosterone in ethanol has a maximum at  $240 \pm 0.25 \text{ m}\mu$ ,  $\epsilon = 16,000$  which is typical of molecules containing the 3-oxo-4-ene grouping. The compound can therefore be detected on paper chromatograms from the absorption of ultraviolet light (mercury line, wavelength  $254 \text{ m}\mu$ ) and  $0.5 \text{ }\mu\text{g.}$  of pure aldosterone in  $0.5 \text{ ml.}$  ethanol (microcell,  $1 \text{ cm.}$  thick) will give a density of about 0.05 at  $240 \text{ m}\mu$  which can be readily measured. However, as with nearly all micro-methods for estimating steroids from biological material, the sensitivity is defined more by the value and variation of the blank obtained than by the reaction of the pure steroid. Eluates of paper or columns tend to have high blank absorption at this wavelength and it is necessary to use control paper areas or column fractions. By measuring the absorption at 235 and  $245 \text{ m}\mu$ , an Allen correction can also be made (Gornall and Gwilliam, 1957). Nevertheless even if this is done variation in the blank values often limits the sensitivity of the method to about  $3 \text{ }\mu\text{g.}$  The method seems to be particularly undesirable for the urinary assay of aldosterone (released from the 3-oxo conjugate) after fractionation of the free compound only, as most of the contaminants described by Nowaczynski *et al.* (1956) absorb ultraviolet at  $240 \text{ m}\mu$  (cf. also Dyrenfurth and Venning, 1959) (Section V, B, 1). However, it may be that formation and chromatography of the diacetate will eliminate these impurities before application of the ultraviolet method (Garst *et al.*, 1960) (Table IV, Method Q).

#### 2. Formazan Formation

The reaction of a steroid containing an  $\alpha$ -ketol side chain with blue tetrazolium [3,3'-dianisol-bis-4,4'-(3,5-diphenyl)tetrazolium chloride] at room temperature gives rise to a formazan which absorbs at  $510 \text{ m}\mu$  in ethanol (Mader and Buck, 1952) and  $560 \text{ m}\mu$  in pyridine. The position of

these maxima may vary with the purity of the reagent (Nowaczynski *et al.*, 1955). The sensitivity for pure steroids is of the same order as for the ultraviolet absorption method and again blank variation often reduces this to about 3  $\mu\text{g}$ . Dyrenfurth and Venning (1959) have compared the blank values obtained after elution from various paper chromatographic systems and have found the Bush B5 to give the lowest values (2  $\mu\text{g}$ . / 8 cm.<sup>2</sup>). The following methods seem suitable.

*a. Method of Nowaczynski et al. (1955).* Tetramethylammonium hydroxide solution was prepared on the day of the reaction by adding 1 ml. of 10% aqueous reagent to 9 ml. of 95% ethanol. To the dried extract was added 0.5 ml. of 95% ethanol, followed by 0.1 ml. of the 1% tetramethylammonium hydroxide solution, and then 0.1 ml. of 0.5% (w/v) blue tetrazolium in 95% ethanol. The solution was then mixed and acidified later with 0.1 ml. glacial acetic acid after being left for 25 minutes at room temperature. The color was stable for 2 hours and the density was recorded at 510  $m\mu$ . A microcell, 10  $\times$  5  $\times$  40 mm., with a 1.5-mm. round slit was used.

The formazan can be developed on paper and eluted with acidic pyridine (Hoffmann and Staudinger, 1951), tetrahydrofuran (Hoffmann and Staudinger *et al.*, 1952), or ethyl acetate-methanol (Touchstone and Hsu, 1955). The merits of such a method have been discussed by Cope (1960) but it has not yet been used for the assay of aldosterone itself. However, it is part of a method for the estimation of tetrahydroaldosterone, a metabolite which retains the  $\alpha$ -ketol grouping.

*b. Method of Liddle et al. (1961); cf. Section V, C2.* The area of the paper was passed evenly and quickly through 0.03% blue tetrazolium salt in 1 *N* sodium hydroxide. The color was developed for 5 to 10 minutes. The sheet was then sprayed with 10% acetic acid and allowed to become almost dry. The areas containing formazan and appropriate paper blanks were eluted with 5 ml. of 10% HCl in pyridine. The paper filaments in the pyridine solution were centrifuged out and the absorbance of the supernatant read at 500, 560, and 620  $m\mu$ . An Allen type correction was used to obtain the density due to the formazan at 560  $m\mu$ .

The formazan can also be estimated directly on paper without elution either by visual comparison (Nowaczynski *et al.*, 1955) or by more quantitative methods of densitometry (Bush and Willoughby, 1957; Bush, 1960c). The first type of estimation is often carried out in assays of aldosterone before the development of soda fluorescence. The whole procedure will be described in the next section.

The specificity of the method is intrinsically low as other groupings such as the 3-oxo-4-ene also reduce the tetrazolium salt albeit weakly,

e.g., progesterone reacts appreciably. However, none of the contaminants described by Nowaczynski *et al.* (1956) react with blue tetrazolium intensely (Section V, B, 1) (Fig. 1).

### 3. Soda Fluorescence

After reaction of a steroid containing the 3-oxo-4-ene group with alkali, the product, under anhydrous conditions and on irradiation with ultraviolet light, fluoresces with an emission spectrum of maximum wavelength of about 560 m $\mu$ . For assays of aldosterone the reaction is usually carried out directly on paper although a method in solution has been published using potassium *tert*-butoxide (Abelson and Bondy, 1955). This has recently been applied for the assay of aldosterone by Staub *et al.* (1961). For direct reaction on paper, after spraying or dipping with a solution of sodium hydroxide, the chromatogram is then treated in different ways by various investigators. Ayres *et al.* (1957a), and Brooks (1960) heat the paper for a short time before drying. Neher and Wettstein (1956), Bush (1960a), Neher (1960), Gowenlock (1960), and Flood *et al.* (1961) recommend leaving the paper for a longer time, at a range of temperature from 20° to 90°C. The following procedure has been found to be satisfactory.

a. *Method of Flood et al. (1961)*. Aliquots of one-third and two-thirds of an extract were applied to separate points on a paper chromatogram (Whatman No. 2, 9 in. wide) as two spots. Two standards of aldosterone (either free or as diacetate) were chromatographed concomitantly and also small amount of dye (F 14 for free compound, F 11 for diacetate, Ciba dyes) running at approximately the same speed as the standards which were placed on the edge of the paper parallel with the origin. An area of paper 9 by 7 in. was then taken with the blue dye in a central position and clamped in a stainless steel frame. Both sides of the paper were sprayed with a total volume of 20 ml. 0.005% blue tetrazolium in 10% aqueous sodium hydroxide. After comparing the formazan reaction of the standards and unknown spots visually, which takes about 5 minutes, the paper was placed in a saturated water vapor atmosphere at room temperature for 1 hour. It was then dried in a forced draft oven at 55°C. for 20 minutes. The soda fluorescence of the unknowns could then be compared with the standards on viewing the paper with an ultraviolet lamp. Most investigators place the accuracy of this type of estimation (two unknowns and two standards) at about 25%. It could also be measured by a fluorimeter in the following manner.

b. *Method of Ayres et al. (1957d)*. The paper was irradiated with ultraviolet light which was filtered to eliminate all but the 365-m $\mu$  mercury line. The emitted light was then filtered to pass light in the

region of a wavelength of 600 m $\mu$ . This particular secondary filter gave the highest signal-to-background ratio with reasonable sensitivity. Unlike most fluorescent methods the incident and emitted light could be in the same direction as, after spraying with blue tetrazolium solution, the paper largely absorbed the ultraviolet light. A template, which was rectangular in shape with a circular aperture straddled with intersecting cross wires, was placed on either side of the paper central to the fluorescent spot and the position fixed with positioning pins. The paper was then cut to this shape and the other spots and a blank area were dealt with in the same way. The pieces were then placed in paper holders and the fluorescence measured. The reading for the blank was subtracted from those of the fluorescent spots and then

$$\frac{\text{unknown total amount of steroid}}{\text{known total amount of standard steroid}} = \frac{\text{sum of two galvanometer readings of unknown amounts}}{\text{sum of two galvanometer readings for standard}}$$

and in this way the readings were weighed approximately according to their reciprocal coefficients of variation. Gowenlock (1960) and Sobel *et al.* (1959) report similar procedures using modifications of commercial instruments, Hilger H 700 Uvispek and Photovolt, Model 540. Another instrument, the Turner fluorimeter available from Macallister Bicknell, Boston, Massachusetts can also be used. Ayres *et al.* (1957d) found that the blank values were equivalent to  $0.5 \pm 0.05$  (S.D.)  $\mu\text{g.}$  of aldosterone diacetate, the coefficient of variation of the over-all assay (two standards, 1 and 0.5  $\mu\text{g.}$  of aldosterone diacetate and two unknowns of about the same amount) was 6% and the response was linear if the spot was equal to or less than 2 cm. in diameter and 6  $\mu\text{g.}$  in amount. Gowenlock (1960) and Flood *et al.* (1961) using slightly different methods of developing the fluorescence at a lower temperature have obtained similar results except that the blank values were found to be lower (about 0.25  $\mu\text{g.}$ ). Brooks (1960), after running the steroid on strips, reported that the blank values were equivalent to  $0.2 \pm 0.03$  (S.D.)  $\mu\text{g.}$  of aldosterone diacetate, but the linearity of the response was restricted to the range 0–4  $\mu\text{g.}$  only, probably due to the steroid being concentrated into a smaller area. These data indicate that 0.5  $\mu\text{g.}$  of steroid (run in two spots with two standards) can be measured to about 15% accuracy and Bartter *et al.* (1960) (Method K, Table III) have applied the method for the assay of adrenal venous blood at even greater sensitivity (about 0.25  $\mu\text{g.}$  total). On scanning the soda fluorescence and radioactivity of 0.04  $\mu\text{g.}$  H<sup>3</sup>-aldosterone on a paper chromatogram, Tait and Tait, 1960, observed good correlation of the two parameters. It seems therefore that

by using a scanning device, which is an accessory in the Turner fluorimeter, the sensitivity could be increased to 0.1  $\mu\text{g.}$  (about 30% coefficient of variation).

The specificity of this reaction for a particular steroid grouping, i.e., the 3-oxo-4-ene (Neher, 1959) is extremely high. However, other compounds which contain this structure such as the contaminants described by Nowaczynski *et al.* (1956) may still interfere with the assay of aldosterone and the specificity of methods using the reaction must still depend on previous chromatographic separation. Although the presence of impurities giving blue fluorescent spots, which are common in biological extracts (von Euw *et al.*, 1959), may interfere with the visual estimation, the use of a yellow filter (Bush and Willoughby, 1957) reduces this as does the secondary filter in the fluorimeter method. The method does not seem to be particularly sensitive to the quenching effects of impurities (Ayres *et al.*, 1957d), although this has been investigated only following column chromatographic purification of the extract before the final paper chromatogram.

#### 4. 2,4-Dinitrophenylhydrazine Method

Gornall and MacDonald (1953) and Gornall and Gwilliam (1957) reported that after reacting aldosterone with 2,4-dinitrophenylhydrazine for 5 minutes at 20°C., the hydrazone had an absorption maximum of 450  $\text{m}\mu$  which is given by all 3-oxo-4-enes. After heating for 90 minutes at 59°C., the maximum shifts to 460  $\text{m}\mu$  and the extinction coefficient is approximately doubled. This is partially due to the formation of a hydrazone at the 20 position. However, as deoxycorticosterone has a lower extinction coefficient at this wavelength there is a possibility that the 18-aldehyde also reacts. Whatever the reason the absorption spectrum after heating is rather different from other similar steroids and this has been the basis of an assay method. The following procedure has been used to measure aldosterone isolated from urine.

*Method of Moolenaar (1957).* The residue was dried and dissolved in 0.1 ml. of ethanol and 0.1 ml. of 2,4-dinitrophenylhydrazine solution [200 mg. 2,4-dinitrophenylhydrazine (recrystallized from *n*-butanol) in 100 ml. of 4 *N*  $\text{H}_2\text{SO}_4$  prepared fresh every day] added. The reaction was allowed to proceed by heating for 25 minutes in a boiling water bath. The insoluble product was coprecipitated by addition of 0.1 ml. 7% sodium benzoate solution in water. The precipitate was washed twice with 2 ml. of a washing solution (1 *N* sulfuric acid, saturated with benzoic acid to which 1 drop/100 ml. of a 5% solution of Teepol was added) followed by centrifugation. The washed precipitate was dissolved in 1 ml. alcoholic sodium hydroxide solution (3 gm. NaOH in

100 ml. 50% ethanol). The extinction of this solution and standard aldosterone was then recorded at 460 and 500  $m\mu$  and then

$$\frac{\mu\text{g. aldosterone in extract}}{E_{460}^{\text{standard}} - E_{500}^{\text{standard}}} = \frac{E_{460} - E_{500}}{E_{460}^{\text{standard}} - E_{500}^{\text{standard}}} \times \text{standard amount in } \mu\text{g.}$$

Aldosterone has a maximum absorption at about 460  $m\mu$  under these conditions. Cortisone and cortisol have a maximum at 480  $m\mu$ ; therefore this calculation produces a zero value for these steroids and reduces the influence of nonspecific chromogens. It is claimed that washing the precipitate eliminates the color from control blank eluates.

Dyrenfurth and Venning (1959) have used the 2,4-dinitrophenylhydrazine method as described by Gornall and Gwilliam (1957). They compared the blank values obtained after various paper chromatographic procedures and found the  $F_2B$  system to be the best in this respect.

#### 5. Method of Lewbart and Mattox (1960, 1961)

Mattox and Lewbart found that in the presence of copper ions and oxygen, the  $\alpha$ -ketol side chain is converted to the 20-ketone, 21-aldehyde grouping which gives an absorption maximum at 410  $m\mu$  in the Porter-Silber reaction (phenylhydrazine-sulfuric acid). This reaction might be expected to be more specific than that with tetrazolium salt as groups which reduce slightly, such as the 3-keto-4-ene, will not react with the Porter-Silber reagent. It may be particularly useful for the estimation of tetrahydroaldosterone (Melby, 1960) for which the soda fluorescence reaction cannot be employed.

Steroids were dissolved in 0.05 ml. methanol, 0.05 ml. of 0.005  $M$  cupric acetate in methanol (1 mg./ml.) added and the mixture left to stand for 50 minutes in air at room temperature. The Porter-Silber reaction was then carried out with the usual reagents but at room temperature for 45 minutes. The reaction with the 20-keto-21-aldehyde group was relatively fast and a maximal density was obtained after 30 minutes. Aldosterone had a maximum absorbance ( $\epsilon = 19,500$ ) at 400  $m\mu$ . Paper blank was 0.3  $\mu\text{g. eq./30 cm.}^2$  Four tenths to eight micrograms of aldosterone could be estimated.

#### 6. Formation of Salicyloyl Hydrazone (Chen, 1959)

Chen reports that 3-oxo-4-enes react with salicyloyl hydrazide to form characteristic hydrazones.

To the dried steroid is added 0.1 ml. salicyloyl hydrazide solution (13.6 mg. in 10 ml. ethanol), 0.01 ml. glacial acetic acid, and 0.5 ml. ethanol. This is heated for 30 minutes at 60°C. and then evaporated to dryness. The residue is then chromatographed on the Bush B5 system

when the aldosterone-salicyloyl hydrazone remains on the origin. The origin is cut out, covered with 2.5 ml. 50% aqueous ethanol and heated briefly. The solvent is decanted into a cell and the absorbance measured at 270, 295, and 320  $m\mu$  and an Allen correction applied to the peak density at 295  $m\mu$ .

This method has been applied to the assay of aldosterone in urine (Chen, 1959).

### 7. Labeled Reagent Methods

The use of labeled reagents for microanalysis has become general in biochemical applications. Keston *et al.* (1949) used  $I^{131}$ -pipsyl chloride (*p*-iodobenzenesulfonyl chloride) to determine amino acids. In this version of the method complete recovery of the derivative was required.

*a. Labeled Derivative as Indicator.* This disadvantage was partially overcome by Keston *et al.* (1950) who added a known quantity of the  $S^{35}$ -labeled pipsyl derivative of the particular amino acid after the preparation of the  $I^{131}$ -pipsyl derivative. The isotopes could be reversed depending on the specific activity required and whether it was preferred to have the short-lived isotope ( $I^{131}$ , 8 days) in the reagent or indicator. In the double isotope technique a value for the amount of amino acid initially present could be calculated from a measurement of the  $S^{35}/I^{131}$  or  $I^{131}/S^{35}$  ratio. This method (Bojeson, 1956, 1958; Bojeson and Degn, 1960) has been applied to the analysis of cortisol and aldosterone in blood. In this case,  $I^{131}$  indicator and  $S^{35}$  reagent were used.

$C^{14}$ -Acetic anhydride was used to detect and aid in the elucidation of the structure of aldosterone (Simpson and Tait, 1952; Simpson *et al.*, 1954a) and (Avivi *et al.*, 1954).  $H^3$ -Acetic anhydride has been successfully used to estimate cortisol in plasma (Avivi *et al.*, 1954) but early attempts to use this reagent to measure aldosterone in peripheral blood failed because of the presence of impurities on the final chromatogram. Kliman and Peterson (1960) have successfully employed  $H^3$ -acetic anhydride to measure aldosterone in urine and adrenal venous blood. In this method aldosterone diacetate ( $C^{14}$ -carboxyl) was added as indicator after acetylation of the original extract with  $H^3$ -acetic anhydride. The advantages of this method are several:

(i) Such methods have an almost limitless potential sensitivity, depending only on the specific activity of the reagent. This can be high enough so that  $10^{-6}$   $\mu g.$  of the original material can be detected. In practice, the sensitivity is limited by the amount of background radioactivity in the final measurement. These labeling methods differ from those using inert reagents in that only impurities reacting with the



labeled reagents and subsequently not separated from the required steroid derivative can contribute to the blank values. Thus precautions such as the use of specially purified solvents are not necessary following the preparation of the derivative.

(ii) Every estimation is unaffected by losses in the procedure after the indicator is added. Other methods depend on a series of control experiments for their estimate of recoveries. The automatic correction for losses in the isotope dilution method also allows the use of highly effective but wasteful purification procedures. This advantage is common to the application of isotope dilution methods to all assay procedures but is particularly necessary in labeled reagent methods where rigorous and extensive fractionation is required.

(iii) The purity of the derivative can be tested in every estimation by a survey of the isotope ratios obtained after the final separation procedure.

(iv) If the labeled derivative is added as the indicator, its specific activity need not be high as it does not react with the reagent.

The method has certain disadvantages:

(i) The method has little intrinsic specificity as any compound containing acylable hydroxyl groups will react with the labeled reagent. The specificity of the results, therefore depends almost entirely on the fractionation procedures used, both before and after the preparation of the derivative. Although the purity of the derivative can be rigorously tested, there may be cases in the measurement of very small amounts of material in biological fluids when the method may prove to be unsuitable because of the presence of labeled impurities in the final sample (Avivi *et al.*, 1954). Svendsen (1960) has described some typical difficulties in the application of the pipsyl method to the measurement of estrogens in plasma. Generally it has been found that it is necessary to chromatograph more than one derivative of the assayed hormone before the final isotope ratio is constant.

(ii) The method is expensive and laborious and requires specialized equipment.

(iii) When the indicator added is a labeled derivative, recovery is only allowed for following the reaction and therefore must be quantitative until after this stage. This will generally require great excess of reagent which is usually expensive and the steroid cannot be fractionated extensively before the reaction.

(iv) Bojesen (1960) reports that if the reagent is not destroyed immediately after the esterification, it may react with trace amounts of free steroid added together with the indicator. In this case, the criterion of

uniform isotope ratio will not indicate that a large blank reading exists. For this reason, even in method (b), carrier should not be added until the reagent is removed.

*b. Labeled Free Steroid as Indicator.* Some of these disadvantages may be overcome by the initial addition of labeled hormone, of negligible weight, to the extract. The specific activity of the indicator steroid must be extremely high as it will react with and be measured by the labeled reagent and the amount added must be subtracted from the final estimate. If the quantity added is high the error of the estimate of the steroid initially present would be impractically large.  $C^{14}$ -Aldosterone could be added for the application of the  $H^3$ -acetic anhydride and  $I^{131}$ -pipsyl reagent methods. In the latter case reagent would have to be prepared or supplied at monthly intervals. The shelf life of the  $H^3$ -acetic anhydride is about 3 months. However,  $C^{14}$ -aldosterone is not yet generally available and the maximum specific activity eventually to be offered is likely to be about 20 mc./millimole. This would require the addition of about 5 m $\mu$ g. of  $C^{14}$ -aldosterone giving 50 c.p.m. (counting efficiency 50%) for a method with an over-all recovery of 20%. This would limit the sensitivity of the method routinely to about 25 m $\mu$ g. and maximally to 10 m $\mu$ g. but would allow the use of the inexpensive reagent  $H^3$ -acetic anhydride (20  $\mu$ mole per reaction at 400 mc./millimole would cost \$7). 7- $H^3$ -Aldosterone of 20  $\mu$ c./ $\mu$ g. specific activity, is generally available and for a method with the same recovery but with the usual  $H^3$  counting efficiency of 15%, 0.2 m $\mu$ g. of added aldosterone would give 250 c.p.m. and 0.5 m $\mu$ g. of the hormone could be measured. This is adequate sensitivity even for the assay of the hormone in human peripheral blood (3 m $\mu$ g./10 ml. plasma). Recently, [1,2]- $H^3$ -aldosterone of specific activity, 100  $\mu$ c./ $\mu$ g., has been prepared (Laumas and Gut, 1961) which increases the potential sensitivity of the method even more. As the reagent,  $S^{35}$ - or  $I^{131}$ -pipsyl or  $C^{14}$ -acetic anhydride could be used. However, their specific activities would have to be about 40  $\mu$ c./ $\mu$ mole to achieve this sensitivity and assuming 20  $\mu$ mole of the reagent can be used for the reaction, the cost per determination would be \$60, 5, and 5 for  $C^{14}$ -acetic anhydride,  $S^{35}$ - and  $I^{131}$ -pipsyl reagent, respectively. These are the current prices from commercial sources when purchased in a reasonable quantity (100 mc.). The prices would be considerably lowered if the reagents were made in the laboratory. For the estimation of larger amounts of aldosterone the specific activity and cost of the reagent could be correspondingly lowered although for routine use it might be more convenient to retain the higher specific activity and reduce the counting time for the  $C^{14}$  assay.

The use of labeled reagents other than acetic anhydride and *p*-iodo-

benzenesulfonyl chloride would be attractive and this possibility remains to be explored.

*c. Measurement of Isotopic Ratios.* (i) *Directly on paper.* In the application of the derivative indicator methods the  $H^3/C^{14}$  or  $S^{35}/I^{131}$  ratios must be measured. The  $S^{35}$  and  $I^{131}$  can be readily assayed and resolved directly on a paper chromatogram. The  $I^{131}$  ( $\beta$ -ray energies, 0.61 and 0.34 mev.) is assayed with a mica window counter and an aluminum filter (about 0.056 mm.) which absorbs completely the  $S^{35}$   $\beta$ -rays (energy 0.17 mev.). The  $S^{35}$  plus  $I^{131}$  is then assayed by removing the filter. The amounts of the individual isotopes present can then be calculated (Svendson, 1960). The simplicity of this measurement is one of the advantages of the pipsyl method carried out in this form. Usually, however, after direct assay on paper it is necessary for the derivative to be eluted before measurement of the ratio in order to obtain greater accuracy.

(ii) *Liquid scintillation counting.* The  $C^{14}/H^3$  ratio is more usually obtained by liquid scintillation counting. The radioactive steroid is dissolved in a phosphor solution. The resultant number of light quanta emitted per  $\beta$ -ray is greater for  $C^{14}$  than for  $H^3$ . Hence the pulse size of the output of the photomultiplier detectors will be different for the two isotopes and they can be resolved by counting at different amplifier and/or discriminator settings. Better characteristics are obtained by leaving the discriminator settings fixed and varying the pulse size by altering the high voltage on the photomultiplier tubes (Tricarb) or the over-all amplification (Tracerlab). The following is a typical procedure:

The steroid was dissolved in 10 ml. of phosphor solution (4 gm. diphenyl oxazole and 100 mg. POPOP per liter toluene) which was placed in a low potassium glass vial (Wheaton Glass, New Jersey). After cooling, the vial was counted in a Tricarb Model 314DC with discriminator settings of 10 to 50 volts. At different high voltages on the photomultipliers the following efficiencies for  $C^{14}$  and  $H^3$  were found: 1140 volts, 14.1%  $H^3$  and 9.1%  $C^{14}$ , 828 volts, 0.63%  $H^3$  and 56%  $C^{14}$  and 745 volts, 0.006%  $H^3$  and 47.1%  $C^{14}$ . The respective backgrounds were 28, 10, and 10 c.p.m. The Tracerlab characteristics are very similar; 1140 volts represents a setting which was just above the balance point (above maximum reading) for  $H^3$ . This point was chosen because the  $C^{14}$  efficiency was considerably reduced without much lowering of the tritium counts; 828 volts represent a position just below the balance point for  $C^{14}$ . By counting at two of these positions the counts of both isotopes can be calculated by the solution of a simultaneous equation. The principles and errors of this type of assay have been discussed (Okita *et al.*, 1957; Tait and Williams, 1952; Kliman and Peterson, 1960).

The error depends on the relative and absolute quantities of  $C^{14}$  and  $H^3$  present but if at least 2000 c.p.m. are obtained at each position then the coefficient of variation of the ratio is of the order of 6%. If the lowest voltage (745 volts) is chosen for measuring  $C^{14}$  the calculations are simplified because the  $H^3$  efficiency is negligible. However, this setting is considerably below the balance point for  $C^{14}$  which leads to greater instability. Nevertheless it may be necessary to count at this voltage when the  $H^3/C^{14}$  ratio of the sample is high.

The Tricarb machine (Model 314 DC) provides a two-channel system which when the lower gate (10–50 volts) is operated to count mainly  $H^3$ , an upper gate assays mainly  $C^{14}$  and therefore as two scaler units are provided the two isotopes can be measured simultaneously. However, because the upper gate has only one variable discriminator (lower level), and because of the characteristics of the amplifier, the measurement of  $C^{14}$  is inaccurate unless large amounts of isotope are present. Modifications of both the Tricarb and Tracerlab machine (separate amplification for both channels), however, are now available which enable the characteristics detailed above for various high-voltage positions to be operated simultaneously and this should halve the counting time or increase the sensitivity of the usual procedure.

Quenching corrections must occasionally be carried out by the addition of an internal high-activity standard after counting the sample. The sample plus standard are then recounted and from a knowledge of the activity of the standard alone, the quenching correction can be calculated. If quenching of both  $H^3$  and  $C^{14}$  is observed, it is simpler to assay a duplicate sample and add a  $H^3$  standard to one, and a  $C^{14}$  standard to the other, although this can be carried out on the same sample by adding the standards in turn. It is advisable to run a standard with all assays and it is most important that this should be prepared exactly as for the other samples. Some steroids tend to adsorb onto glass when dissolved in the pure toluene which is used for liquid scintillation counting. It is therefore advisable to add 2% ethanol either to the stock solution of scintillator or to the sample. As this causes slight quenching, it is important to add this to the standard solution also and to count the standard at the same electronic settings.

## B. METHODS OF IDENTIFICATION

It is often desirable in assays of aldosterone to check the identity of the material in the final purified extract. In addition to the reactions previously described, which are suitable for quantitative estimations and

should agree with one another, there are other more qualitative tests which can also be carried out on a microscale.

### 1. Infrared Spectroscopy

Infrared spectroscopy of the free compound in chloroform or potassium bromide disk may be performed. With suitable techniques (N. R. Jones and Sandorfy, 1956), the absorption spectrum in the carbonyl region can be obtained with 5  $\mu$ g. steroid. The carbonyl peaks in chloroform at 6.25 and 5.98  $\mu$  are typical of those found with steroids having the 3-oxo-4-ene grouping and that at 5.86  $\mu$  is due to the unconjugated 20-keto group. The weak extinction of this latter peak is the only distinguishing feature. Because of hemiacetal formation, there is no peak due to the 18-aldehyde (Simpson *et al.*, 1954b).

### 2. Sulfuric Acid Chromogen

The absorption spectrum developed by leaving aldosterone in concentrated sulfuric acid for 2 hours at 20°C. has a single peak at 288  $m\mu$ . After heating the same solution for 1 hour at 90°C., peaks are obtained at 247, 285, 342, and 460  $m\mu$ . This test could be carried out on 6  $\mu$ g. steroid in 0.5 ml. sulfuric acid (Simpson *et al.*, 1954a).

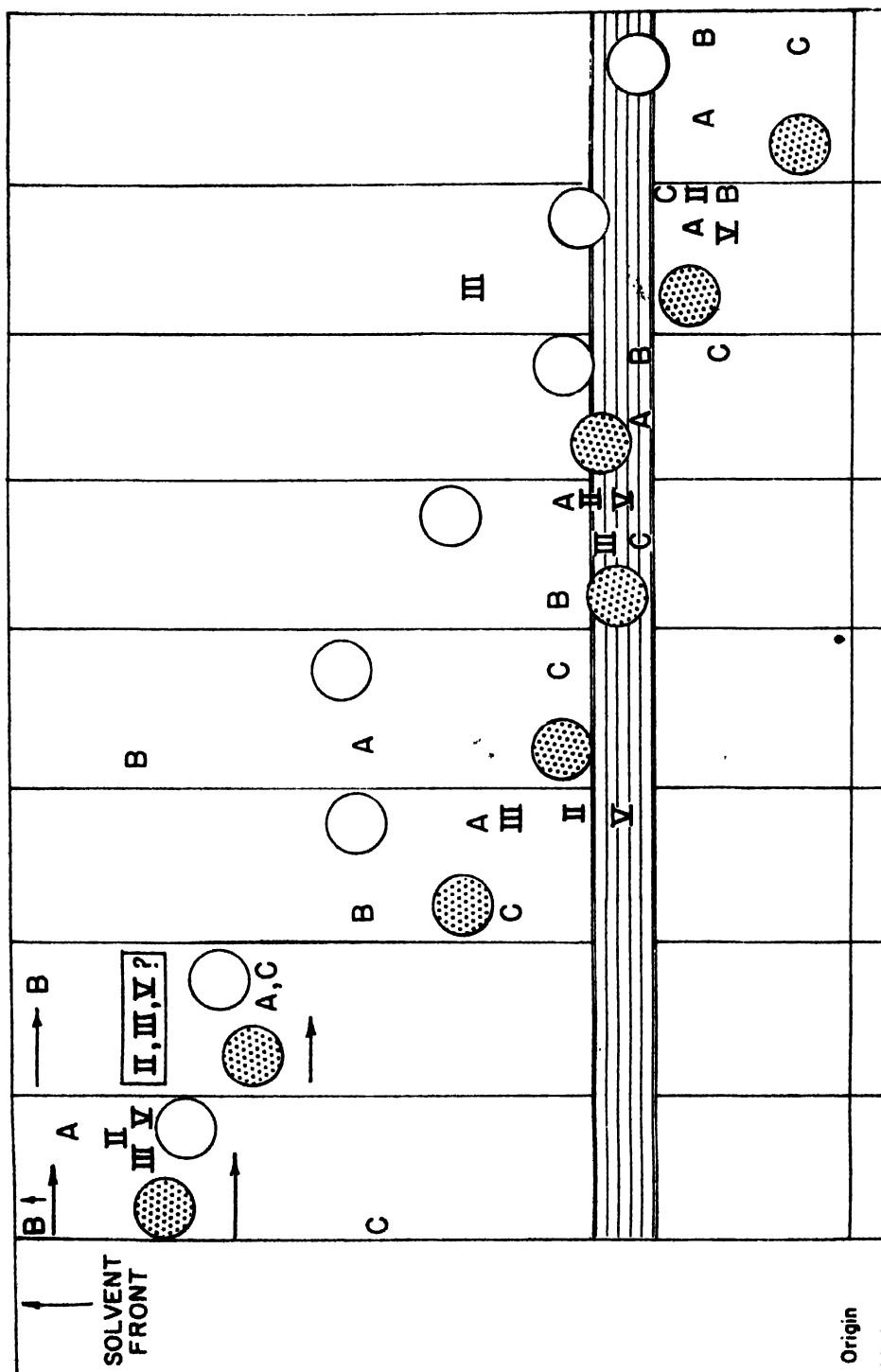
### 3. Phosphoric Acid Chromogen

After heating aldosterone in a solution of 3 ml., 100% phosphoric acid [79 gm. phosphorous pentoxide (Bakers) in 100 gm. 85% phosphoric acid (Mallinckrodt)] for 20 minutes at 107°C., the absorption spectrum of the product has a single peak at 280  $m\mu$  (Nowaczynski and Steyermark, 1956). This can also be carried out with 6  $\mu$ g. steroid but because of its high viscosity, the reagent has to be handled at 100°C.

### 4. Negative Tests

Most steroids on paper chromatograms, after being treated with 15% phosphoric acid according to the method of Neher and Wettstein (1951), fluoresce on being viewed with ultraviolet light of a wavelength about 360  $m\mu$ . However, aldosterone gives no fluorescence and this reaction may be used as a test for impurities (Simpson *et al.*, 1954a; Baulieu *et al.*, 1956). Five micrograms of cortisol and corticosterone give brilliant fluorescence.

The phenylhydrazine-sulfuric acid (Porter and Silber, 1950) or sulfuric acid/phosphoric anhydride (Steyermark and Nowaczynski, 1955) reactions in solution or the Zimmermann reaction in solution or on paper (Bush and Willoughby, 1957) can also be used to detect the presence of






Solvent Systems	P.G. - Tol.	Form./CHCl <sub>3</sub>	B5	Form./Benz.	C	Cy-Di	E <sub>2</sub> B	F/BBW
	Propylene glycol - toluene	Formamide chloroform	Benzene - methanol - water (2:1:1)	Formamide - benzene	Toluene - ethyl acetate - methanol - water (9:1:5:5)	Cyclohexane - dioxane - ethanol - water (4:4:2:1)	Isocitane - t-butanol - water (10:5:9)	Formamide: butyl acetate - n-butanol - water (85:15:5)
Approximate running time	50% p.g. in methanol at 22°C., 96 hrs. Ethyl glycol - tol. 50% in methanol at 24°C., 48 hrs.	30% formamide in acetone at 22°C. 3 hrs.	24° or 37°C 3 hrs.	30% formamide in acetone at 24°C., 24 hrs.	22° or 37°C 2 hrs.	38° 16 hrs.	22 or 37°C., 8 hrs.	20% formamide in acetone at 22°C., 3 1/2 hrs.
References	Zaffaroni et al (1950) cf also ethyl glycol - toluene Nowaczynski et al (1958)	Zaffaroni (1953)	Bush (1952) cf also similar systems Moolenaar (1957) Singer & Stack-Dunne (1955)	Zaffaroni et al (1950) Burton et al (1951) Zaffaroni & Burton (1951)	Bush (1952) Bush (1954)	Chen et al (1958) Neher & Wettstein (1960)	Eberlein - Bongiovanni (1954)	Mattox & Lewbart (1959) as modified by Neher & Wettstein (1960)
		Cortisol		Aldosterone		Cortisone	II Nowaczynski-Genest Compounds III (IV) V (not as in Fig. 2)	
A - 18, 21-dihydroxy-pregn-4-ene-3,11,20-trione (18 hydroxy A) IX, Fig. 2			B - 21-hydroxy-pregn-4-ene-3,11,20-trione - 18 al. (18 aldo A) X, Fig. 2			C - 17 iso (side chain configuration) aldosterone XI, Fig. 2		

Fig. 1. The running properties of aldosterone and possible contaminants in various paper chromatographic systems. Approximate *R<sub>f</sub>* values taken from Neher (1959), Neher and Wettstein (1960), Nowaczynski *et al.* (1956, 1957, 1958), and Genest (1961). Compound IV travels with compound III in all systems where the position of compound III is indicated.

impurities. Aldosterone gives a negative test with all these methods. It is of interest in this connection that 18-hydroxydeoxycorticosterone, and therefore perhaps certain other 18-hydroxy  $\alpha$ -ketols, gives a positive Porter-Silber reaction (Péron, 1961). Thus tetrahydroaldosterone gives a negative reaction but the contaminant 3 $\alpha$ ,18,21-trihydroxy-5 $\beta$ -pregnane-11,20-dione reacts positively.

#### 5. Paper Chromatography of the Free Steroid

No other compound, either of known or unknown identity, has been reported which runs at the same speed as aldosterone in the seven paper chromatographic systems shown in Fig. 1. As low as 0.25  $\mu$ g. of aldosterone can be detected as a single spot by soda fluorescence and therefore a complete investigation of these running properties can be carried out with 2  $\mu$ g. of the hormone.

#### 6. Formation of Derivatives

*a. Diacetate.* The 18,21-diacetate (compound III, Fig. 2) can readily be produced from aldosterone in quantitative yield. The free steroid is dissolved in 0.3 ml. of pyridine, and 0.15 ml. of acetic anhydride is added. The mixture is left for 15 hours at 20°C. The solution is then evaporated, 1 ml. of methanol added and taken to dryness *in vacuo*. The product can then be characterized by its running properties in various paper chromatographic systems (Table II), e.g., in the propylene glycol-toluene system the diacetate runs at the same speed as 11-dehydrocorticosterone monoacetate (Simpson *et al.*, 1954a). It can usually be detected by tetrazolium and soda fluorescence reactions or radioactivity if C<sup>14</sup> or H<sup>3</sup>-acetic anhydride is used as reagent (Simpson and Tait, 1952). The diacetate is only weakly active in most bioassays but the free compound may be regenerated by hydrolysis with potassium bicarbonate (Simpson *et al.*, 1954a) or enzyme (Mattox *et al.*, 1953a; Farrell *et al.*, 1954). The infrared spectrum of the diacetate dissolved in carbon disulfide shows the expected peaks in the carbonyl region due to the 3-oxo-4-ene and 20-oxo groups. The acetoxy peaks in this region are superimposed perhaps due to the proximity of the 18 and 21 groups. The sulfuric acid chromogen of the free aldosterone and its diacetate should be identical.

*b. 11,18-Lactone 21-Monoacetate.* Oxidation of the diacetate with chromic acid (Section V, B, 4) gives a unique product, which can be identified by its running properties in the Cy-Di system of Kliman and Peterson (1960) ( $R_{\text{deoxycortisol}} = 1$ ). Presumably this is the 11,18-lactone 21-monoacetate of aldosterone (compound VI, Fig. 2) which is also produced by chromic acid oxidation of aldosterone 21-monoacetate



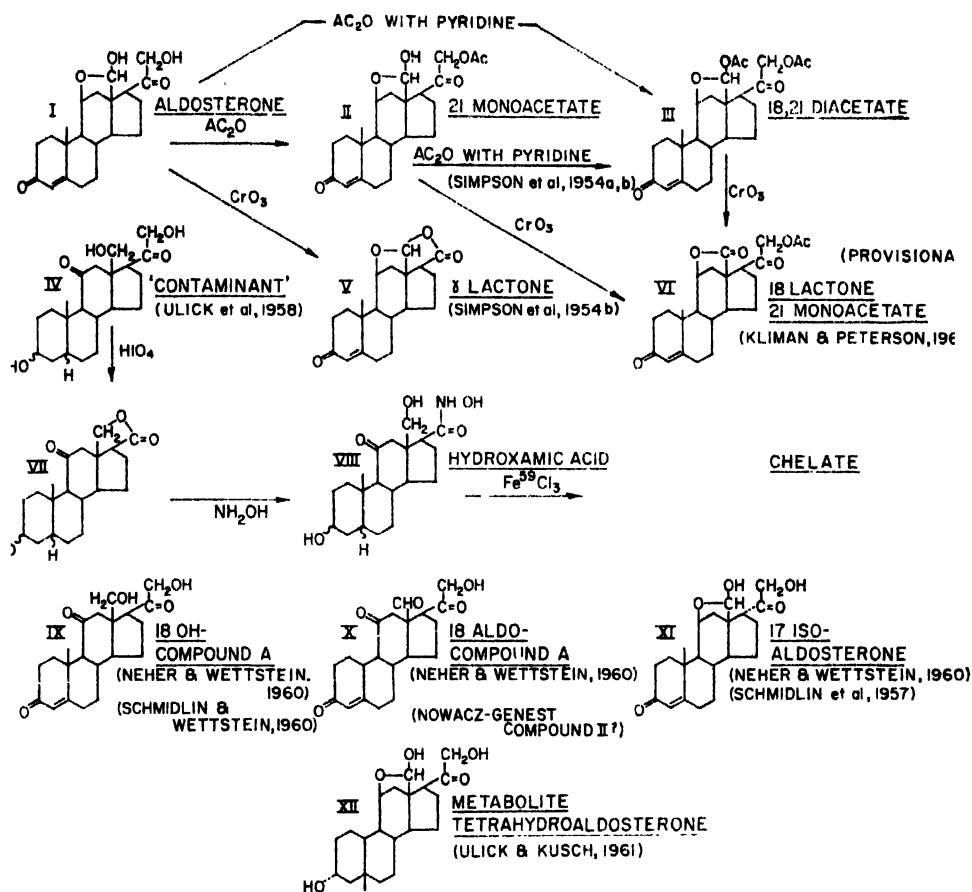


Fig. 2. Compounds of interest in assay methods for aldosterone.

(Simpson *et al.*, 1954b) and runs at the same speed as 11-dehydrocorticosterone monoacetate in the propylene glycol-toluene system. This is a useful derivative for identification purposes and for applications of the labeled reagent method (Kliman and Peterson, 1960) but the yield on oxidation of the diacetate is not quantitative.

*c.  $\gamma$ -Lactone.* This compound (V, Fig. 2) is readily formed by chromic or periodic acid oxidation of aldosterone (Simpson *et al.*, 1954b). Aldosterone was dissolved in 0.7 ml. methanol, and 1.3 ml. sodium periodate solution was added (1.25 gm.  $\text{Na}_2\text{H}_3\text{IO}_6$  was suspended in 70 ml. of distilled water and 2 *N* sulfuric acid, 3.85 ml. was added until the salt dissolved and the pH was 3–4. The solution was finally made up with water to 100 ml., about 10 mg.  $\text{Na}_2\text{IO}_4$  per milliliter). After 2 hours the solution was evaporated to dryness *in vacuo*. Sulfuric acid (2 *N*) was added until the pH was approximately 1 and this was extracted 3 times with

TABLE II  
RUNNING PROPERTIES OF DERIVATIVES OF ALDOSTERONE AS EMPLOYED FOR THE ASSAY OF THE HORMONE

Compound	<i>R</i> value in terms of solvent front or reference compound						
	Aldosterone diacetate	Front	Front	Adrenosterone	21-Deoxy-cortisone	21-Deoxy-cortisone	Acetate
Adrenosterone	—	0.23	—	1.00	—	1.20	—
11-Dehydrocorticosterone acetate	—	—	—	—	—	—	1.00
Corticosterone acetate	1.0	0.5	—	—	—	—	—
21-Deoxycortisone	—	—	—	—	1.00	1.00	—
Aldosterone diacetate	1.0	0.5	0.62	1.00	—	1.03	0.95
18-Lactone of aldosterone 21-monoacetate	—	—	—	—	1.00	—	—
Aldosterone 21-monoacetate	—	0.04	0.23	—	—	—	—
Compound V acetate	—	—	0.38	—	—	—	—
Compound III acetate	0.10	0.09	0.28	—	—	—	—
Solvent systems (all at 23°):	A	B3	B1	Cy-Benz. 4:2	Cy-Benz. 4:3	Cy-Di	P.g.-hexane
	Petroleum ether, b.p. 80-100°C-methanol-water (4:4:1)	Petroleum ether, b.p. 80-100°C-benzene-methanol-water (2:1:4:1)	Petroleum ether, b.p. 80-100°C-toluene-methanol-water (1:1:7:3)	Cyclohexane-benzene-methanol-water (4:2:4:1)	Cyclohexane-benzene-methanol-water (4:3:4:1)	Cyclohexane-dioxane-methanol-water (4:4:2:1)	Propylene glycol-hexane
Approximate running time (hours):	16	3	3	16	17	18	72
References:	Bush (1952) Brooks (1960)	Bush (1952) Ayres <i>et al.</i> (1957 a-d) Sobel <i>et al.</i> (1959)	Bush (1952) Genest (1960) Moolenaar (1957)	Kliman and Peterson (1960)	Kliman and Peterson (1960)	Kliman and Peterson (1960)	Garst <i>et al.</i> (1960)

chloroform-ether (1:3 v/v) which was then washed with aqueous solutions. The solvent was then taken to dryness *in vacuo*.

The yield of the lactone under these conditions is quantitative. It can be identified by its running properties in the Bush B5 ( $R_{\text{adrenosterone}} = 1$ ) (Simpson *et al.*, 1954b) and formamide-cyclohexane-benzene (1:1 v/v) (6.5-hour running time, lactone 19.3 cm. and adrenosterone 22.3 cm.) (Dyrenfurth and Venning, 1959) systems using soda fluorescence as the method of detection. The infrared absorption spectrum has a peak at  $5.6 \mu$  in the carbonyl region due to the lactone in addition to the usual 3-oxo-4-ene peaks (Simpson *et al.*, 1954b). This derivative is stable and sublimates easily. It may therefore be useful in gas chromatographic methods.

Tetrahydroaldosterone (XII, Fig. 2) can also be identified by the formation of the corresponding lactone under these oxidation conditions (Ulick and Kusch, 1961).

For an examination of the accuracy of a quantitative assay, it is necessary to check whether all the final density, fluorescence, or radioactivity is due to aldosterone itself. Therefore, if a derivative is formed from the final extract during the investigation, it is advisable to apply an isotope dilution method to correct for recovery. An example of such an investigation, as applied to the specificity of a plasma cortisol method, has been described by Peterson *et al.* (1957).

### III. Methods of Purification

None of the reactions described with the possible exception of some bioassay procedures have great specificity and the accuracy of most methods will therefore depend entirely on the efficiency of the preceding purification.

#### A. INITIAL PURIFICATION

The degree of initial purification required will depend on the nature of the analyzed biological material and the capacity and properties of the later higher resolution systems. The capacity of these subsequent systems for steroids and certain impurities is in descending order: column partition chromatography, paper chromatography with impregnated stationary phase such as the Zaffaroni *et al.* (1950) and Mattox and Lewbart (1959) systems (Fig. 1), and the type of paper chromatography, where the stationary phase is applied by equilibration, such as those described by Bush (1952) and Eberlein-Bongiovanni (1954). How-

ever, because certain polar impurities such as phospholipids may affect the resolution of column partition systems, particularly when they are to be reused, initial purification is advisable.

The first stage in an assay usually consists of the extraction of aldosterone from an aqueous phase with methylene dichloride or chloroform. The partition coefficient of aldosterone (concentration in solvent/concentration in water) (Flood *et al.*, 1961) in these systems is 33.0 and 27.2, respectively. From the studies of Burstein (1956) on the partition coefficient of  $C_{21}O_5$  and  $C_{21}O_6$  steroids in aqueous chloroform and ethyl acetate solvent systems, it might be expected that ethyl acetate would be a superior solvent. However, not only is the partition coefficient of aldosterone in chloroform/water (27.2) rather better than expected (cortisol 9.5) but the ethyl acetate/water coefficient is considerably worse (aldosterone 3.8, cortisol 15.1) (Underwood *et al.*, 1961). Therefore, ethyl acetate is not the preferred solvent for aldosterone as it is for other steroids with the same number of oxygen atoms. It may, however, be the solvent of choice for the extraction of tetrahydroaldosterone. This has not yet been thoroughly investigated. The exact procedures employed will depend in an *ad hoc* manner on the biological material and whether aldosterone or a metabolite is being measured. They will be detailed individually in the later sections. In some cases, the extracted material is now sufficiently pure for it to be applied directly to the column (Tait *et al.*, 1961b) or on the paper (1 mg. per centimeter, 30 mg. total) in Zaffaroni systems (Brooks, 1960). In most cases, however, further solvent partition, silica gel adsorption chromatography, or single-phase paper chromatography is required.

### 1. Solvent Partitioning

The extract can be purified by partition between water and benzene (10:1 v/v). The water is then extracted with chloroform (Baulieu *et al.*, 1956).

The extract can also be dissolved in 1.5 ml. ethanol; then 5 ml. water is added and the mixture washed with 3 volumes cyclohexane. The aqueous phase is extracted with 7 volumes dichloromethane (Kliman and Peterson, 1960).

### 2. Silica Gel Chromatography

Adsorption chromatography of the extract on neutral and relatively inactive silica gel has been the most frequently used method of initial purification. It is quick, convenient and efficient and recoveries are excellent. The silica gel from most commercial sources e.g., Davison can be used without further treatment for this purpose.

a. *Method of Bush and Sandberg (1953)*. A slurry of 3 gm. of silica gel (Davison, mesh size 60–200) was made with petroleum ether [b.p. 80–100 (Skellysolve C)]–ethyl acetate (1:1 v/v) mixture and poured into a glass column, 1 cm. in diameter, with a total of 15 ml. solvent. The dried extract was then put onto the silica column with a total of 18 ml. of the same solvent in 4 washings. The corticosteroids were then eluted with a total of 8 ml. methanol-ethyl acetate (1:1 v/v); 0.01  $\mu$ g. labeled cortisol and aldosterone in an extract were eluted from this column with a recovery of over 95%.

b. *Method of Neher and Wettstein (1955)*. The crude extract was dried dissolved in chloroform and put onto a 2.5 gm. silica gel (Davison, 200 mesh) column, prepared as a slurry in  $\text{CHCl}_3$  (ratio of height to diameter of the column about 4). It was then washed with 50 ml. chloroform-acetone (99:1 v/v) and eluted with 100 ml. chloroform-acetone (1:1 v/v).

### 3. Single-Phase Paper Chromatography

This method was introduced into the steroid field by Bush (1952). It involved spotting the extract along a lane, 1 cm. in width, and running an ascending chromatogram with a single phase (ethyl acetate-chloroform, 1:1 v/v). The steroids move on the solvent front and are finally concentrated into small rectangles on the origin of the subsequent partition chromatogram. Lipids and certain pigments remain along the lane and the procedure is essentially reverse phase chromatography. The advantage of this procedure is that it combines initial purification with the application of steroid to the paper, which has to be carried out in any case if the subsequent partition chromatography is on this material. It can also be applied to several extracts simultaneously but has lower capacity than silica gel column chromatography. One disadvantage is that steroid is occasionally irreversibly lost by adsorption in this procedure.

a. *Method of Bush Applied by Gowenlock (1960)*. The extract containing aldosterone was spotted along a lane and ascending chromatography, using in this case ethyl acetate-methanol (1:1 v/v), was carried out to concentrate the steroid at the distal end of the strip. The same paper is then used for the subsequent partition chromatography in a Bush system.

b. *Method of Schmidt and Staudinger (1954) Applied by Dyrenfurth and Venning (1959)*. A tank, containing 1 liter of water saturated with heptanol and 300 ml. heptanol in a separate container, was used for the purification. The extract was applied along 17 cm., normally to the

direction of solvent flow, on Whatman paper No. 1 (washed with distilled water). Twenty-five milliliters of water saturated with heptanol were then run down the paper for 5 hours after equilibrating for 3 hours. Urinary pigments and lipids were left at the origin and the steroids eluted for the subsequent chromatography in Zaffaroni systems. Dyrenfurth and Venning (1959) claim that this reverse phase method has advantages over silica gel chromatography and solvent partitioning.

## B. PAPER AND COLUMN PARTITION CHROMATOGRAPHY

Many different paper and column partition systems have been used for the fractionation of aldosterone (Fig. 1), its derivatives (Table II) and metabolite, tetrahydroaldosterone (Section V, C) in assay methods. The Zaffaroni and Mattox-Lewbart systems use paper impregnated with formamide or propylene glycol as the stationary phase and benzene, chloroform, toluene, or a mixture of butyl acetate, *n*-butanol, and water as the mobile phase. The Bush, Eberlein-Bongiovanni and Kliman and Peterson (1960) paper systems employ aqueous mixtures (water with methanol, ethanol, or butanol) as the stationary phase and benzene or toluene alone or with petroleum ether (b.p. 80–100°C.) or ethyl acetate (Bush), cyclohexane with benzene or dioxane (Kliman and Peterson, 1960) or isooctane *tert*-butanol (E<sub>2</sub>B) as mobile phase. The aqueous phase is applied in the conventional manner by prior equilibration of the paper with the vapor of the stationary phase. Although the Zaffaroni systems have been used on partition columns for the preparation of aldosterone (Mattox *et al.*, 1953a, b), only the Bush solvent systems have been used on columns for assay methods.

### 1. Principles

The ability of a partition system to separate two steroids will depend on the difference of partition coefficients of the compound in the solvent system and the resolution of the chromatographic procedure. This resolution depends on a constant (number of theoretical plates in a fixed length of column or number of theoretical plates per centimeter of steroid movement on paper, which is an intrinsic property of the system and can be experimentally determined). It is also dependent on the manner in which the procedure is operated. Thus the over-all resolving power can be defined as the minimum fractional difference in the partition coefficients of two steroids which would result in their separation (2.3% mutual contamination) in the particular system (Tait and Tait, 1960). Then, on this basis, the resolving power

$$= \frac{\text{theoretical plate value}}{\sqrt{\text{theoretical plate value}}} \times \frac{1}{\sqrt{1 - R}}$$

where

$$R = \frac{\text{movement of position of maximum concentration of solute}}{\text{simultaneous movement of solvent front in either column or paper methods}}$$

Therefore the higher the theoretical plate value and the lower the  $R$  value, the better the separation of the steroids. This is particularly so as diffusion effects are negligible in most partition systems (Tait and Tait, 1960). It is therefore preferable to operate paper systems with low  $R_f$  values and long running distances of the steroids. Under these conditions, for a steroid run for 25 cm. at low  $R_f$  values, the theoretical plate value is about 900. When operated at an  $R_f$  of 0.4, and when the solvent front is run to the end of the paper, the resolution of a paper system is reduced about three times. The resolution is also reduced when the extract contains large amounts of impurities or steroids. In this case the steroid spreads appreciably and a large cut must be taken to ensure quantitative recovery. Thus when a paper system is used for the first separation, the equivalent number of theoretical plates is only about 100. This could be improved by the application of isotope dilution techniques, but probably not by more than a factor of two. The theoretical plate value of a 60-cm. partition column is about 900 and this is not greatly affected by the quantity of steroids and impurities chromatographed. Theoretically, therefore, on this basis only, the optimum combination would be partition column followed by paper chromatography of the purified extract. This is the basis of the methods devised by Ayres *et al.* (1957c) and Flood *et al.* (1961).

However, the potential resolution of the systems at a fixed difference in partition coefficients is not the only parameter to be considered in devising methods of purifying a steroid. It may be more efficient, provided that the resolutions are reasonable, to use solvent systems in which the partition coefficients of aldosterone and potential impurities are as different as possible. Thus, as can be seen from Fig. 1, the propylene glycol-toluene or chloroform-formamide systems could be used to separate aldosterone from cortisol and the Bush C, E<sub>2</sub>B, and the Mattox-Lewbart system for removing cortisone. This is the basis of the method of Neher and Wettstein (1955, 1956) which employs a Zaffaroni system followed by the Bush C and also the methods of Nowaczynski *et al.* (1957), Mattox and Lewbart (1959), and Dyrenfurth and Venning (1959) for purifying aldosterone from cortisone, cortisol, and other contaminants.

Formation and chromatography of derivatives of aldosterone may be a quicker and more convenient method of purification than either very high resolution or multiple chromatography of the free hormone. It may also be combined with these methods. The diacetate, which can be reproducibly and quantitatively made, has been the most popular derivative for this purpose (Table IV). When particularly good fractionation is required as in the application of labeled reagent methods it may be necessary to form several derivatives. A masterly theoretical treatment of the running properties of derivatives of steroids has been given by Bush (1960b).

## 2. Elution

After partition column chromatography, the steroid is automatically eluted and if benzene or toluene solvent systems are used an aliquot may be added directly to liquid scintillation phosphor solutions for counting with little quenching. Also many reactions for the assay of aldosterone can be applied directly to the final paper chromatogram and elution is not required. However, if multiple paper chromatography is used to purify the aldosterone, elution of the steroid from the paper between stages must be carried out. This can be done either by direct extraction of the paper or by single-phase chromatography.

*a. Method of Neher and Wettstein (1955): Direct Extraction.* The paper was cut up and shaken with 20% methanol to form a thick pulp. After standing for  $\frac{1}{2}$  hour, the pulp was filtered and slowly washed twice with methanol. The filtrate was evaporated to about  $\frac{1}{2}$  volume and shaken 3 times with  $\frac{1}{2}$  volume chloroform, which was taken to dryness. The residue was then extracted with acetone. The recovery was 90%. Dyrenfurth, Venning (1959), and Garst *et al.* (1960) cut the paper into small pieces and wash several times with methanol.

*b. Method of Neher and Wettstein (1955): Single-Phase Chromatography.* The appropriate areas of the paper strips were attached to 5-cm.-broad syphons made of two sheets of aluminum and packed with glass wool. The end of the syphon away from the paper was put into a shallow dish filled with 80% methanol. Before the solvent front reached the lower edge of the paper strip, a pointed piece of paper saturated with 80% methanol was attached to quicken drainage of the solvent (total volume 15 ml.) although this is not absolutely necessary. The recovery was 95%. Steyermark (1955) also uses a capillary device, similar to that described by Haines (1952), for putting the solvent (25 ml. of 80% methanol) onto the paper. It is essential that the flow of solvent be slow enough so that chromatography occurs. Recovery was 95%. Another piece of apparatus, operating on the same principle, has been described by



Brooks (1960) using a total of 4 ml. 95% ethanol. Recovery was 90%.

In general, the chromatography type of method is to be preferred as the recovery is higher and blank values lower for the same volume of solvent. However, when the method is combined with a partition step, as in (a) above, the blank values may be lower than for the simple chromatographic procedure (b) (Neher, 1961). It seems preferable, in order to get high recoveries, to choose a solvent which is miscible with the stationary phase and to avoid drying the paper completely before elution. It is clear that for methods involving more than two chromatographic steps the elution recovery must be greater than 90% unless isotope dilution is used.

Washing of the paper is usually not required for direct reaction on paper to develop formazan or soda fluorescence. However, it may be necessary before elution and reaction in solution (Nowaczynski *et al.*, 1955; Dyrenfurth and Venning, 1959).

### C. OTHER METHODS

Perri and co-workers (1960) have separated aldosterone from biological extracts by two reversed phase paper chromatographic systems. The advantages of this type of procedure are not yet clear.

The total time taken to equilibrate and run paper and column solid-liquid chromatograms is considerable. Various other methods have been or are being developed to quicken the time of separation of steroids.

Staub and co-workers (1961) have described a method for measuring aldosterone in urine which employs two chromatograms of the free steroid and two of the diacetate. All these separations are carried out on  $\text{KH}_2\text{PO}_4$ -impregnated glass fiber paper after initial purification by silica gel chromatography. The steroid is finally estimated by the potassium *tert*-butoxide reaction (Abelson and Bondy, 1955). The advantage of this procedure is the short time required for one chromatogram (total time 15 minutes). The disadvantage appears to be the limited capacity of the systems.

Gas chromatography of steroids is being rapidly developed (Vanden Heuvel and Horning, 1960). Although aldosterone itself is probably too sensitive for the high temperatures required, suitable derivatives will probably be found (e.g., diacetate or  $\gamma$ -lactone). The advantages of the procedure would be speed (about 20 minutes) and automatic detection with resolution of the same order as liquid-solid column chromatography with the possibility of much greater resolution as the art progresses. The disadvantage appears to be low capacity, bearing in mind the lack of specificity of the present sensitive detectors. This procedure would appear

to have great possibilities when coupled with the double isotope-labeled reagent methods. Thin layer adsorption chromatography is another method (Kirchner *et al.*, 1951) which has speed (45 minutes per chromatogram) but also good capacity. Its resolution for steroids remains to be investigated but our own group has had promising results with derivatives.

#### IV. Metabolism of Aldosterone in Man

A discussion of the metabolism of aldosterone in man seems to be an essential preliminary for an appreciation of clinical methods of assay of the hormone.

##### A. PATTERN OF URINARY METABOLITES IN NORMAL SUBJECTS

Flood *et al.* (1961) have studied the excretion of radioactivity after the simultaneous intravenous injection of tracer amounts of 4-C<sup>14</sup>-cortisol (10  $\mu$ g.) and 7-H<sup>3</sup>-aldosterone (0.1  $\mu$ g.) into five normal women. In the first 48 hours after injection, over 90% of both the administered cortisol and aldosterone was excreted in the urine and this appears to be the predominant normal route of excretion of the metabolites of these hormones in man. The amount of radioactivity released by  $\beta$ -glucuronidase from these urines was also similar for both steroids (56% of the injected dose for aldosterone compared with 64% for cortisol). As is already well established for cortisol, the urinary excretion of conjugates of glucuronic acid is therefore the major route of metabolism of aldosterone in normal subjects. In contrast, the amount released by extraction of the urine by chloroform at pH 1 for 24 hours at room temperature was very different for the two hormones. About 12% of the injected aldosterone and 2% of the cortisol was extracted by this procedure. In the case of cortisol, none of this released radioactivity was due to the hormone itself whereas about one-half of the total tritium activity after acid hydrolysis was contributed by the aldosterone (about 6% of the injected dose). After correcting for losses and inefficiency of hydrolysis, it has been estimated that the parent conjugate (previously termed the 3-oxo conjugate of aldosterone; see K. M. Jones *et al.*, 1959) represents about 14% of the secretion in normal subjects and is therefore one of the major metabolites of the hormone (Flood *et al.*, 1961; Underwood *et al.*, 1961). The term "3-oxo conjugate" is an unsatisfactory one and should be replaced when the structure is known. A more general term, however, such as the aldosterone conjugate would give rise to confusion in a review dealing with several metabolites. "3-Oxo conjugate" does not imply that a conjugation

is on the 3 position but only that the 3 position is not irreversibly reduced. Hydrolysis of the 3-oxo conjugate, followed by purification and measurement of the released aldosterone, has been the basis of most clinical assay methods since the classic studies of Luetscher (1956), Venning *et al.* (1954), and Gordan *et al.* (1954). The 3-oxo conjugate is not extracted from urine either by chloroform or ethyl acetate (Underwood *et al.*, 1961), hence if initial extraction at neutral pH is carried out before acid hydrolysis, the free steroid present but not the 3-oxo conjugate can be removed, and hence the conjugate specifically estimated (Rinsler and Rigby, 1957; K. M. Jones *et al.*, 1959). Otherwise a mixture of free aldosterone plus 3-oxo conjugate will be measured and although in normal subjects the excretion of unconjugated aldosterone is negligible (about 0.2% of the injected dose) theoretically there may be pathological conditions where this is not the case.

Recent work has confirmed previous conclusions (K. M. Jones *et al.*, 1959) that the 3-oxo conjugate has a high renal clearance or is formed in the kidney, as it is not present in blood in significant amounts (Peterson, 1959) and over 95% of the radioactivity in the form of the metabolite is excreted within 12 hours of an injection of labeled aldosterone (Flood *et al.*, 1961). These investigators have also studied the excretion of radioactivity in 0- to 24- and 24- to 48-hour collections of urine after the injection of labeled cortisol and aldosterone. Most of the glucuronide metabolites of aldosterone were excreted in the first 24 hours. However, relative to the corresponding metabolites of cortisol, the excretion of aldosterone glucuronides was slightly delayed. This confirms the data of Laragh *et al.* (1960), on the rate of excretion of the major metabolite of aldosterone conjugated with glucuronic acid which indicated that, in urines of subjects with normal kidney function, radioactivity in the form of this conjugate requires 48 hours for complete (>95%) excretion. This difference in the rate of excretion of the 3-oxo conjugate and the glucuronide metabolites may be of some importance in the choice of metabolite for an assay method. If it is desirable to measure a metabolite whose excretion responds quickly to any change in the secretion of the hormone, e.g., investigation of diurnal rhythm (Muller *et al.*, 1958) then the 3-oxo conjugate should be chosen. On the other hand, if an estimate of the mean secretion over 2 days is required, e.g., diagnosis or investigation of primary aldosteronism and hypertension (Laragh *et al.*, 1960), the measurement of the glucuronide metabolite might be preferable.

Ulick and Lieberman (1957) purified a compound from urine after incubation with  $\beta$ -glucuronidase which they concluded was 3 $\xi$ ,18,21-trihydroxy-5 $\xi$ -pregnene-11,20-dione (IV, Fig. 2). This was first detected, following hydrolysis, by oxidation to a  $\gamma$ -lactone (VII, Fig. 2). The  $\gamma$ -lactone

was converted to a hydroxamic acid (VIII, Fig. 2) which was then chelated with  $\text{Fe}^{59}$  (Fig. 2). This method, employed in the earlier work by Ulick and co-workers, has given way to one using  $\text{C}^{14}$ -acetic anhydride although the first approach seemed to be of great promise for the assay of aldosterone and its metabolites. Although the properties of the oxidation product were consistent with the proposed structure other characteristics of the original compound reported such as the immediate reaction with tetrazolium and the ready formation of a triacetate were surprising in view of the behavior under corresponding conditions of other 18-hydroxyl compounds (Neher and Wettstein, 1960). Flood *et al.* (1961) also found that the major metabolite of aldosterone conjugated with glucuronic acid formed a triacetate and reacted readily with tetrazolium. Both Ulick and Lieberman (1957) and Flood *et al.* (1961) concluded that the compound they were measuring was a metabolite of aldosterone because the triacetate was radioactive after an injection of tritiated aldosterone in man. The problem has been clarified by some recent work of Ulick and Kusch (1961). They have shown that the material purified by Ulick and Lieberman was a mixture containing  $3\alpha,18,21$ -trihydroxy- $5\beta$ -pregnene-11,20-dione and the A-ring reduced aldosterone  $3\alpha,11\beta,21$ -trihydroxy- $5\beta$ -pregnane-20-one-18 al, henceforward termed tetrahydroaldosterone (compound XII, i.e. Fig. 2). The former compound (IV, Fig. 2) oxidizes to XII (Fig. 2) but reduces tetrazolium slowly and forms a diacetate. It is also not radioactive after injection of tritiated aldosterone and appears to be a metabolite of 18-hydroxycorticosterone not of aldosterone. Tetrahydroaldosterone (compound XII) forms a triacetate and reduces tetrazolium rapidly. A similar metabolite was detected in human peripheral blood (Ayres *et al.*, 1957b). A preparation of rat liver also converts aldosterone to tetrahydroaldosterone ( $3\alpha,5\beta$ ) (Ulick *et al.*, 1961). A crude homogenate of the same tissue was earlier found to form the  $3\beta,5\alpha$  stereo isomer (Pechet *et al.*, 1960). Tetrahydroaldosterone (compound XII, Fig. 2) is radioactive after injection of tritiated aldosterone in man and is the major metabolite conjugated with glucuronic acid. Fortunately the methods devised by Ulick and coworkers involve the formation of the triacetate and hence the contaminant, compound IV, which is not a metabolite of aldosterone would not be measured. Simpler methods involving chromatography of the free compound only will probably give results which are too high because of the presence of compound IV, unless, as in the method of Flood *et al.* (1961) radioactivity is the only parameter measured as will be described.

Flood *et al.* (1961) examined the pattern of radioactive compounds released by  $\beta$ -glucuronidase as revealed by partition column chromatography. About 75% of the total tritium radioactivity was due to a single

peak running between tetrahydrocortisone and tetrahydrocortisol. This peak was consistently responsible for about 35% of the injected dose in normal women. Very little tritium could be detected in the more polar fractions of the column which, from the chromatographic behavior of corresponding metabolites of cortisol, suggests that aldosterone is metabolized to 3,20-hydroxyl compounds (analogous to the cortols and cortolones) to a minor extent only (Fukushima *et al.*, 1960; Romanoff *et al.*, 1957; Bush and Willoughby, 1957). The major radioactive peak (henceforward referred to as compound  $\alpha$ ) was homogeneous on chromatography in various systems and by the same criteria appeared to be identical with the tetrahydroaldosterone of Ulick and Kusch (1961), (compound XII, Fig. 2). The only discrepancy in the characteristics of compound  $\alpha$  (Flood *et al.*, 1961) and those described by Ulick and co-workers (Ulick *et al.*, 1958; Laragh *et al.*, 1960) for their compound (measured as the triacetate) lies in the reported proportion of the injected hormone excreted. The latter group estimate 10–15% as tetrahydroaldosterone whereas the former give 35% as compound  $\alpha$ . However, it should be noted that the lower value was obtained in studies which did not require an accurate measurement of the total amount excreted. Liddle *et al.* (1961) find that, using a method similar to that described by Ulick but correcting carefully for losses throughout purification, 40% of the injected dose is tetrahydroaldosterone. This is in agreement with the results of Flood *et al.* (1961). There is no conclusive evidence that either the compound measured by the method of Ulick and co-workers or compound  $\alpha$  is a single entity but it would appear that if several compounds are present they must be very closely related. This is not taking into account compound IV, Fig. 2, which apparently is not radioactive after injection of tritiated aldosterone nor does it form a triacetate and would therefore not be measured by either method.

Nevertheless, according to the studies of the Taits and co-workers, the over-all pattern of the metabolites of aldosterone conjugated with glucuronic acid is a remarkably simple one, 75% of the total being due to a single or very closely related group of compounds and no other single compound accounting for over 3% of the injected dose. Therefore, the 3-oxo conjugate and compound  $\alpha$  together comprise about one-half of the total metabolites of aldosterone, at least in normal subjects, and determination of these two compounds would be expected to be adequate for most clinical investigations where an estimate of the production of the hormone is required.

By injecting labeled aldosterone and measuring the radioactivity in various urinary fractions (Ayres *et al.*, 1957b; Peterson, 1959; Ulick *et al.*, 1958) it has been shown that the pattern of metabolism is unaltered

when a subject is placed on a low salt diet although this results in an increase in the excretion of the 3-oxo conjugate (Luetscher, 1956). The pattern of metabolites also remained unchanged at least as with respect to total 3-oxo conjugate and glucuronide after intramuscular administration of 10 mg. per day *d*-aldosterone for 4 days to a normal subject (Rosemberg, 1961). This demonstrates, as was first pointed out by Axelrad *et al.* (1955) from the good inverse correlation between sodium excretion and aldosterone excretion (free aldosterone plus aldosterone from 3-oxo conjugate) in various subjects, that in most physiological and many pathological investigations, the measurement of either metabolite will suffice to indicate the production rate of the hormone. Unlike those of cortisol, the pattern of the known urinary metabolites of aldosterone has not yet been shown to be dependent on the concentration of the hormone. More detailed investigations may, of course, alter this conclusion. A comparison of the clearance rate of aldosterone at different plasma concentrations of the hormone has not yet been made.

#### B. ABNORMAL METABOLISM

There are now several clinical conditions where it is established that the pattern of metabolism may change. This may be indicated in a particular group of patients by a lack of inverse correlation between sodium output and the excretion of a particular metabolite of aldosterone as in pregnancy and in acute hepatitis although this may not be the only reason for this effect (e.g., salt-losing nephritis) (Buchborn *et al.*, 1957; Ayres *et al.*, 1957a). K. M. Jones *et al.* (1959) found a marked alteration in the pattern of urinary metabolites in pregnancy with an increase in the proportion of 3-oxo conjugate and a decrease in glucuronides. A measurement of either of these metabolites alone would therefore give a misleading indication of secretion rate. Hurter and Nabarro (1960) have reported that in cirrhosis of the liver some patients show an altered pattern of metabolism somewhat similar to that which occurs in pregnancy. They also reported that this pattern can change very markedly from day to day. In one case of Conn's syndrome Ulick *et al.* (1958) found that the secretion rate of aldosterone and excretion of tetrahydroaldosterone is increased but the aldosterone from the 3-oxo conjugate is in the normal range. On the other hand, women treated with Enovid show a normal proportion of 3-oxo conjugate but a decreased percentage of tetrahydroaldosterone (Layne, 1961). There is also some indication of an altered pattern of metabolism of aldosterone in hypertensive disease (Genest, 1960; Laragh, 1960). Although many of these studies are in a preliminary stage, there is now abundant evidence that it is dangerous to rely on the meas-

urement of one metabolite only for an indication of the secretion rate in all clinical situations.

In conditions where the pattern of metabolism may change, the calculation of secretion rates from the specific activity of a single urinary metabolite after the injection of the radioactive hormone (secretion rate equals radioactivity injected divided by the specific activity of the metabolite) has proved to be a simple and powerful *in vivo* method which has the advantage that only the specific activity of one metabolite needs to be analyzed. One of the methods to be described (Ulick *et al.*, 1958) is therefore mainly concerned with this aim (specific activity estimate of tetrahydroaldosterone) without regard to quantitative recovery which is not necessary for the calculation of the secretion rate. If the secretion rate is calculated from the specific activity of one metabolite after a single injection of radioactive hormone, the quantity of another metabolite can be estimated from a knowledge of its radioactivity only. This is the basis of one method (Flood *et al.*, 1961) of measuring compound  $\alpha$  (tetrahydroaldosterone).

With all these approaches available, by a judicious choice and operation of an appropriate method bearing in mind the mode of metabolism in a particular group of patients, a reliable estimate of the secretion rate of aldosterone by the adrenal gland can now be made. Correlation of this value with the general electrolyte and hemodynamic status of the patient provides one criterion for the diagnosis of Conn's syndrome.

## V. Urinary Assay Methods

### A. HYDROLYSIS OF 3-Oxo CONJUGATE

Procedures for the hydrolysis of the conjugate under acid conditions vary widely in the method of contact of the aqueous and organic phases, the length of time for which the hydrolysis is allowed to proceed, and other conditions.

Walraven (quoted by Ayres *et al.*, 1957a) obtained two to three times as much aldosterone by use of continuous extraction (Cohen extractor) of the urine by chloroform at pH 1 for 24 hours compared with leaving the urine at pH 1 without organic solvent. Dyrenfurth and Venning (1957) also reported greater yields for some urines in similar experiments. These reports, together with certain theoretical reasoning have been the basis for the use of continuous extraction by various groups (Liddle *et al.*, 1955; Brooks, 1960; Gowenlock, 1960; Ayres *et al.*, 1957a; K. M. Jones *et al.*, 1959; Flood *et al.*, 1961).

On the other hand, Mattox and Lewbart (1959) found continuous

extraction and methods involving mechanical stirring produced insignificantly greater yields of aldosterone compared with those obtained by leaving the urine at pH 1 without organic solvent present. Kliman and Peterson (1960) also found continuous extraction no more efficient. Luetscher and co-workers (Axelrad *et al.*, 1955; Neher and Wettstein, 1956; Baulieu and De Vigan, 1958; Hernando *et al.*, 1957; Garst *et al.*, 1960) all employ methods which leave the urine undisturbed at pH 1 for 24 hours. Moolenaar (1957) and Sobel *et al.* (1959) use continuous mixing with frequent changes of solvent.

Underwood *et al.* (1961) by measuring the tritium released as aldosterone from urine by acid hydrolysis after injection of 7- $H^3$ -aldosterone, have found very little difference in the yield, either when the urine is left standing with or without chloroform present at pH 1 or whether agitation, shaking with chloroform, or continuous extraction is used. This confirms the conclusions of Mattox and Lewbart (1959) and Kliman and Peterson (1960) that the simple method of leaving the urine undisturbed at pH 1 is preferable to other more complicated methods which do not increase the yield of aldosterone but may give more impurities (Mattox and Lewbart, 1959).

Sobel *et al.* (1959), employing the method of continuous mixing, have studied the release of aldosterone as a function of the period of hydrolysis at pH 1.0. The yield of aldosterone for the second 24 hours of hydrolysis varied between 33 and 82% of that in the first 24 hours. However, the pH of the urine was measured as 1.0 by a test paper method. Dyrenfurth and Venning (1957), working with pregnancy urine at pH 1.5, obtained rather similar results. Kliman and Peterson (1960) allowed the urine to stand at pH 1 with no organic solvent present and found that the first 24-hour period of hydrolysis produced 7 and 33 times more aldosterone than in the second and third 24-hour periods. Underwood *et al.* (1961) obtained rather similar results. Both groups of workers also report about 10% destruction of aldosterone in 24 hours of acid hydrolysis. These results indicate that a 48 rather than a 24-hour period of acid hydrolysis at pH 1 (measured by a pH meter) will not increase the yield of aldosterone unless the solvent is replenished during the process. Even so, the increase in aldosterone will be only 10% in the second 24 hours and so the extra period of hydrolysis is not necessary except for the most demanding requirements. A pH of 1 appears to be the optimum  $H^+$  ion concentration to use, since it has been shown, Kliman and Peterson (1960), that at both higher and lower values, the amount of aldosterone released in 24 hours is less than at pH 1. At pH 0.5 the rate of aldosterone released from the 3-oxo conjugate is greater than at pH 1 but there is increased destruction. Although this could possibly be reduced by continuous ex-



traction using fresh solvents at frequent intervals (continuous extraction by the Cohen apparatus does not accomplish this), this would require a rather complicated apparatus and in routine laboratory practice, accurate measurement of pH values lower than 1.0 would be difficult to attain.

To summarize, leaving the urine undisturbed at pH 1 (measured by a pH meter) for 24 hours followed by hand extraction with chloroform or methylene dichloride seems to be a satisfactory routine clinical procedure. As previously discussed, prior extraction of the urine at neutral pH to eliminate free steroid would simplify the interpretation of results. It also eliminates most of the cortisol, cortisone, and certain other potential impurities so easing the subsequent problems of purifying the aldosterone released from the 3-oxo conjugate. After the extraction at pH 1, the solvent is usually washed with alkali (sodium carbonate at room temperature or sodium hydroxide at low temperature). Garst *et al.* (1960) report large losses of aldosterone if sodium hydroxide is used without careful control of temperature. The use of sodium carbonate seems preferable in a routine procedure, unless unusual amounts of acidic pigments are present.

#### B. MEASUREMENT OF ALDOSTERONE RELEASED FROM 3-Oxo CONJUGATE

The methods which have been most frequently used are described in Tables III and IV. Except in certain circumstances, such as studies on hypoaldosteronism (e.g., high salt diet), cases of adrenal tumor or subjects on unusual diets (e.g., high intake of citrus fruits), the assay of aldosterone from the 3-oxo conjugate is not a demanding procedure as regards either sensitivity (normal excretion, 10  $\mu$ g. per day) or specificity.

##### 1. *Method of Neher and Wettstein and Modifications*

The relatively simple physicochemical procedure described by Neher and Wettstein (1956) (Method G, Table III) which involves an initial Zaffaroni paper chromatographic separation followed by another on a Bush C system and then estimation of the aldosterone visually after formazan and soda fluorescence development, has proved adequate for many extensive clinical investigations, e.g., Buchborn *et al.* (1957) and Muller *et al.* (1956). It has the advantage that it is readily adaptable to multiple assays. However, several investigators (Nowaczynski *et al.*, 1957; Dyrenfurth and Venning, 1959; Brooks, 1960; Sobel *et al.*, 1959; Mattox and Lewbart, 1959) have reported difficulty in assaying certain urines using this method, mainly due to the presence of impurities on the final chromatogram which cause the soda fluorescence and formazan estimates

TABLE II. METHODS OF ASSAYING ALDOSTERONE IN BLOOD, INCUBATION MEDIUM OR URINE (FROM 3-OXO CONJUGATE) WHICH EMPLOY PAPER CHROMATOGRAPHY OF FREE COMPOUND BEFORE REACTION. COEFFICIENT OF VARIATION =  $\frac{S.E.}{\text{MEAN}} \times 100\%$ . NORMAL VALUES ARE FULLY CORRECTED FOR RECOVERY.

Author	Material	Method of hydrolysis or extraction	Preliminary purification	Chromatographic Methods	Reaction and overall sensitivity	Recovery %	Overall precision (coefficient of variation) %	Specificity	Normal values (corrected for recovery)
A. Genot et al (1959)	Dog adrenal blood	Extraction by $\text{CHCl}_3$	Washed	1 paper as free compound					
A. Mulrow et al (1959)	Rat adrenal incubate			Form. - $\text{CHCl}_3$	Bioassay (Johnson, 1954) 2.0 $\mu\text{g}$	-	about 30	Bioassay	-
B. Das Gupta and Goud (1959)	Rat adrenal blood	Whole blood extracted with ethyl acetate	Partition between light petroleum ether & aqueous methanol (Bush, 1952)	Bush B5	Bioassay (Simpson & Tait 1952) 0.4 $\mu\text{g}$	-	30	Bioassay	-
C. Singer and Sieck-Dunne (1959)	Rat adrenal blood	Whole blood extracted with ethyl acetate	Partition between light petroleum ether & aqueous methanol (Bush, 1952)	Tal - methanol-water (4:5:1) at 37°	Bioassay (Simpson & Tait 1952) 0.4 $\mu\text{g}$	-	30	Bioassay	-
D. Lieberman and Luetscher (1960)	Urine	At pH 1 for 24 hrs. without $\text{CHCl}_3$	Extraction $\text{CHCl}_3$ Washed	Pg. - Toluene	Bioassay (Johnson, 1954) 2.0 $\mu\text{g}$	-	about 30	Bioassay	Uncorrected 0.8 - 7.8 $\mu\text{g}/\text{day}$ (16 subjects) Mean 2.5 $\mu\text{g}/\text{day}$
E. Singer (1960)	Rat adrenal blood	Whole blood extracted with ethyl acetate	Washed	2 papers as free compound Form. - $\text{CHCl}_3$ then Bush C	Soda fluorescence on paper-fluorimeter - 2.0 $\mu\text{g}$	41 ± 6		Initially checked by bioassay	-
F. Stockenius and Giroud (1959)	Rat or rat adrenal incubate	Medium extracted with $\text{CHCl}_3$	Filtered	Bush B5 then Bush C sometimes further E2B	U.V. absorption, visual soda fluorescence & tetrazolium 2.0 $\mu\text{g}$ Bioassay 0.5 $\mu\text{g}$	-	20	Agreement of reactions and bioassay	-
G. Meher & Wettstein (1966)	Urine	pH 1.5 for 24 hrs without $\text{CHCl}_3$	Extraction $\text{CHCl}_3$ washed checked per cent recovery if extracted over 60 mg. (Meher and Wettstein, 1965)	Form. - $\text{CHCl}_3$ then Bush C	Tetrazol. and soda fluorescence on paper - Visual estimate	75	30	Compounds II, III, V, VI, and VII possible contaminants. Also 17 $\alpha$ -OH and 18 $\alpha$ -OH compounds A (Norecyclyl or 1957) (Nishikawa and Wettstein, 1960) Checked by agreement tetrazolium & soda fluorescence	Range 0.7 - 17 $\mu\text{g}/\text{day}$ (p. 605) Mean 6.5 $\mu\text{g}/\text{day}$

H. Boulier & De Vigan (1952)	Urine		pH 1.3 for 24 hrs. without $\text{CHCl}_3$	Extraction $\text{CHCl}_3$ washed Benzene-water partition	2. Paper as free compound Form - $\text{CHCl}_3$ then Bush C Sometimes checked by paper chromatography of diacetate	Tetraz and soda fluorescence on paper: visual estimation checked by phosphoric acid fluorescence	about 75	about 30	As above (Neher and Weinstein 1956) cf also (Mattox & Leubner 1959) and (Brooks 1960)	Range 5-8 $\mu\text{g/day}$ Mean 4 $\mu\text{g/day}$
I. Molesnar (1957)	Urine		Sol. with NaCl pH 1 for 23 hrs over $\text{CHCl}_3$ Discontinuous extraction and shaking	Washed, 70% methanol-tol-perroleum ether 1:1, partition	Tol - N octanol-methanol-water (96:2:50:50), then Bush B1 6 hrs	2,4 dimethoxybenzylhydrazine (Gornall and Weiss 1953) 2.0 $\mu\text{g}$ .	about 70	about 20	Non specific chromogens present. Reference on correction	Range 4-20 $\mu\text{g/day}$ (12 subjects) Mean 8.5 $\mu\text{g/day}$
J. Reich (1958)	Sheep adrenal venous blood		Plasma extracted with ethyl acetate-ether, 2:1	Silica gel chromatography (Bush 1953) Run up on paper (Bush 1952)	Tol-methanol-water (3:3:1), 172 hrs at 50° repeat in same system 5 hrs	Soda fluorescence on paper: estimate 0.5 $\mu\text{g}$ .	90	20	Checked by diacetate formation, tetrazolium in solution	-
K. Berrier et al (1960)	Dog adrenal venous blood		Plasma extracted with $\text{CHCl}_3$	Applied directly	3 papers as free compound Form - benzene- $\text{CHCl}_3$ -water (90:10:5:5); Tol-methanol-water (10:7:3) then Bush C	Soda fluorescence on paper: fluorimeter 0.25 $\mu\text{g}$	42 $\pm$ 9 $^{\circ}\text{C}$ cellulose tested	12	High in blood	-
L. Nowaczynski et al (1957)	Urine		pH 1 at 30 hrs over $\text{CHCl}_3$ Discontinuous extraction during this time Other methods sometimes used including incubation with $\beta$ -glucuronidase	Washed Silica gel chromatography (Neher and Weinstein 1955)	Ethyl alcohol-toluene (48 hrs) then $\text{E}_2\text{B}$ then Bush B5	Ultraviolet absorption, tetrazolium in solution (Nowaczynski et al 1955) checked by phosphoric acid chromogen	80 with extreme care	15 if recovery maintained	High Sobel et al (1959) reports catalysis following B5 occasionally lowers results Cf. also Drydenluth & Vanhing (1959), 4 papers	Range 3-15 $\mu\text{g}$ . (9 subjects) Mean 8.5 $\mu\text{g}$ .
M. Drydenluth and Vanhing (1959)	Urine		pH 1.5 at 24-48 hrs over $\text{CHCl}_3$	Washed N hexanol-water partition chromatography (Schmidt and Steudinger 1953)	Form - $\text{CHCl}_3$ then Bush C then $\text{E}_2\text{B}$	Boassay (Schmidt et al 1955) (8 $\mu\text{g}$ ) U.V. absorption and tetrazolium in solution 2 $\mu\text{g}$	60	Physico-chemical 20	U.V. higher than tetraz. in few cases probably due to Compound V	Range 1.9-18 $\mu\text{g/day}$ (14 subjects) Mean 6.5 $\mu\text{g/day}$
N. Mattox and Leubner (1959)	Urine		pH 1 at 24 hrs without $\text{CHCl}_3$	Extracted $\text{CHCl}_3$ washed	Form - $\text{CHCl}_3$ then form-water-butyl acetate (Leubner and Leubner, 1958) then Bush C	Soda fluorescence on paper - visual estimation 2 $\mu\text{g}$	80	About 25	"use of FBW system eliminates an compound" B-hydroxy and also A. high reported in FBW. Properties of Gasest compounds II - XI unknown	Range 3-20 $\mu\text{g/day}$ (24 subjects) Mean 7 $\mu\text{g/day}$
O. Drydenluth and Vanhing (1959)	Urine		pH 1 at 24 hrs without $\text{CHCl}_3$	Washed N hexanol-water partition chromatography (Schmidt and Steudinger, 1953)	4 papers as free compound P 9 tol, then Bush C then $\text{E}_2\text{B}$ then Bush B5	U.V. absorption, tetraz (2 $\mu\text{g}$ ) solution and boassay (1.8 $\mu\text{g}$ ) (Vanhing et al 1956)	60	Physico-chemical 20	U.V. tetraz and bo-assay agree after B5. Boassay and tetraz agree after $\text{E}_2\text{B}$ but U.V. high (Compound XI) until after B5	Range 1.9-15 $\mu\text{g/day}$ (14 subjects) Mean 6.5 $\mu\text{g/day}$

TABLE 2 METHODS OF ASSAYING ALDOSTERONE IN BLOOD, INCUBATION MEDIUM OR URINE (FROM 3-OXO CONJUGATE) WHICH EMPLOY CHROMATOGRAPHY OF FREE COMPOUND AND/OR DERIVATIVES BEFORE REACTION COEFFICIENT OF VARIATION =  $\frac{S.E.}{\text{MEAN}} \times 100\%$ . NORMAL VALUES ARE FULLY CORRECTED FOR RECOVERY. SENSITIVITY = TOTAL AMOUNT REQUIRED FOR REASONABLE ASSAY.

Author	Material	Method of hydrolysis or extraction	Preliminary purification	Chromatographic methods	Reaction and overall sensitivity	Recovery %	Overall precision (coefficient of variation) %	Specificity	Normal values (corrected for recovery)
				1 paper as free compound 1 paper as diacetate					
P. Ferrel et al (1954)	Dog adrenal venous blood	Whole blood with $\text{CHCl}_3$	Partition between 70% ethanol and hexane	P g - tol (72 hrs) as free compound then p g - hexane as diacetate	U v absorption 3 $\mu\text{g}$ sens Bioassay (after hydrolysis) 2 $\mu\text{g}$ sens	About 70	15	Checked by u v and by hydrolysis (cholesterase) and bioassay	-
O. Gerst et al (1960)	Urine	pH 1 for 24 hrs without $\text{CHCl}_3$	Washed and then applied directly	P g - tol (48-60 hrs) as free compound then p g - hexane as diacetate (22-24 hrs)	U v absorption about 3 $\mu\text{g}$ sens	80	about 15	Not known	Range 6.3 - 18.1 $\mu\text{g/day}$ (18 subjects) Mean 11.3 $\mu\text{g/day}$
				2 paper as free compound 1 paper as diacetate					
R. Sobel et al (1959)	Urine	Continuously stirred at pH 1 over $\text{CHCl}_3$ for 48 hours	Washed silica gel chromatography (Nehar & Wentman 1955)	P g - tol (48 hrs) then E <sub>3</sub> B (6 hrs) as free compound then Bush B3 (6 hrs) as diacetate	Soda fluorescence on paper fluorimeter measurement 2 $\mu\text{g}$ sens	74	12	Occasionally rerunning required High	Range 2.8-20 $\mu\text{g/day}$ (10 subjects) (P OCS) Mean 11 $\mu\text{g/day}$
S. Brooks (1960)	Urine	Continuously extracted at pH 1 with $\text{CHCl}_3$ for 24 hours	Washed silica gel chromatography (Bush & Sandberg 1953) when extract over 30 mg	Form - $\text{CHCl}_3$ then Bush C as free compound then as diacetate in Bush A (16 hrs)	Soda fluorescence on paper fluorimeter measurement 2 $\mu\text{g}$ sens	70	12	High	Range 7-9 $\mu\text{g/day}$ (4 subjects) Mean 8 $\mu\text{g/day}$
				1 column as free compound 1 paper as diacetate					
T. Flood et al (1961)	Urine	Continuously extracted at pH 1 for 24 hrs with $\text{CHCl}_3$	Washed silica gel chromatography (Bush & Sandberg 1953)	Water-methanol, i. l., toluene, Celite 545 on column as free compound then as diacetate in Bush B3 paper chromatogram; 7- $\text{H}$ diesterone added to urine as indicator; peak fractions on first column acetylated	Soda fluorescence fluorimeter measurement 2 $\mu\text{g}$ sens	Automatically corrected throughout procedure 74	8	Correlation of $^3\text{H}$ with soda fluorescence	Range 4.1-10 $\mu\text{g/day}$ (14 subjects) Mean 7.5 $\mu\text{g/day}$

U. Ayres et al (1957); Goswami (1960)	Urine	Continuously extracted at pH 1 for 24 hrs with $\text{CHCl}_3$	Washed silica gel chromatography (Bush and Sandberg, 1953)	1 column as free compound 1 column as diacetate 1 paper as diacetate	Water-methanol 1:1; benzene-ethyl acetate, 5:2 column as free compound Water-methanol 1:4, light petroleum ether-benzene, 3:2 column as diacetate then Bush B5 paper system	Soda fluorescence fluorimeter measurement 2 $\mu\text{g}$ sens	7.7 unit indicator added then recovery corrected arithmetically	15	High No serious impurities in many pathological urines reported by Goswami, 1960	Range 6-30 $\mu\text{g/day}$ (31 subjects) Mean 13 $\mu\text{g/day}$
V. Ayres et al. (1960)	Ox adrenal gland incubate	Extracted $\text{CHCl}_3$	Washed (Bush and Sandberg, 1953)	2 columns as free compound 1 column as diacetate 1 paper as diacetate	Water-methanol 1:1, benzene-ethyl acetate 5:2, Celite 545 and water-methanol 1:1, benzene-ethyl acetate 5:2, light petroleum ether-benzene 3:2 column as diacetate Bush B3 paper as diacetate	Soda fluorescence fluorimeter measurement 2 $\mu\text{g}$ sens	47	15	High Final specific activity uniform in radioactive biosynthetic experiments	-
W Davis et al (1958)	Dog adrenal venous blood	Plasma extracted with $\text{CH}_2\text{Cl}_2$	Dried and acetylated	2 papers as diacetate 1 paper as 18 lactone 21 monoacetate	Acetylation of extract Cyclohexane-benzene-methanol-water (4:2:4:1) and cyclohexane-dioxane-methanol-water (4:4:2:1) paper systems as diacetate After chromic acid oxidation, cyclohexane-benzene-methanol-water (4:3:4:1) paper system as 18 lactone 21 monoacetate	$^3\text{H}$ acetic anhydride used for acetylation $^3\text{H}$ -diacetate as indicator $^3\text{H}$ - $^3\text{H}$ used for estimate 0.01 $\mu\text{g}$ sens	95-100 before indicator added Overall about 20	10	$^3\text{H}$ - $^3\text{H}$ constant on final chromatogram	-
X. Kivinen and Peterson (1960)	Urine	About 30 ml kept at pH 1 for 24 hrs without $\text{CHCl}_3$	Extracted with $\text{CHCl}_3$ and washed, if more than 1/50th of daily output, portion between aqueous methanol and cyclohexane	As above	As above	As above 0.01 $\mu\text{g}$ sens	About 95 before indicator added Overall about 20	12	$^3\text{H}$ - $^3\text{H}$ constant on final chromatogram	Range 5.3-20 $\mu\text{g/day}$ (26 subjects) Mean 10.5 $\mu\text{g/day}$
Y. Bosen and Dagn (1960)	Plasma, peripheral and adrenal	20-30 ml plasma extracted with $\text{CHCl}_3$	Dried and esterified with $^3\text{H}$ -propyl reagent	2 papers as pipson 1 paper as oxidized pipson	Chromatographed ethyl acetate-acid-water and formamide- $\text{CHCl}_3$ paper systems Oxidized chromic acid then formamide- $\text{CHCl}_3$ paper system	$^3\text{H}$ - $^3\text{H}$ ratio about 0.002 $\mu\text{g}$	80% to addition of indicator Automatically corrected following	10	$^3\text{H}$ - $^3\text{H}$ constant on final chromatogram	About 20 $\mu\text{g}/100\text{ml}$ plasma human peripheral-normal diet

to disagree. As Dyrenfurth and Venning (1959) state "The zone containing impurities is excluded when the Neher-Wettstein method is followed precisely. In practice however and when the Zaffaroni system is applied, the cortisone zone and this zone overlap and it is difficult to obtain a clear-cut separation. Therefore it is felt to be safer to include the upper part of this dark zone and rely on subsequent systems for a more complete separation." Nowaczynski *et al.* (1956) have reported a number of compounds which are potential contaminants in the Neher-Wettstein method. Their running properties relative to those of aldosterone and cortisol in various paper systems are shown in Fig. 1. Figure 1 also shows by the position of the horizontal arrows the usual areas of the initial chromatograms (propylene glycol-toluene or formamide-chloroform) which are eluted before the subsequent Bush C chromatogram. Because of the shorter running time required, the formamide-chloroform system is now the preferred first separation. It is likely that the Nowaczynski-Genest compounds would be included in the area cut out in the formamide-chloroform system although their running properties have been reported only in the propylene glycol-toluene system. Compound II, which may be 18-aldo compound A (compound X, Fig. 2), occurs rarely. Compound III which appears to be identical with the material isolated from citrus fruits by Gray *et al.* (1955) is more common and according to Genest and co-workers is excreted in large quantities in subjects on a high potassium diet. Both Nowaczynski *et al.* (1958) and Baulieu and De Vigan (1958) report that compound III is released efficiently by incubation with  $\beta$ -glucuronidase and hardly at all by hydrolysis at pH 1. It appears that the risk of contamination of aldosterone by this compound could be greatly reduced by avoiding enzymatic hydrolysis. Compound IV behaves similarly to compound III. Compound V appears to be the major potential impurity as it is released by hydrolysis at pH 1. Dyrenfurth and Venning (1959), Nowaczynski *et al.* (1957), and Mattox and Lewbart (1959) (Methods M, L, and N, Table III) have devised methods employing three or even four paper separations of the free compound to avoid these impurities. The method of Dyrenfurth and Venning (1959) also eliminates a salt excretor which appears in pregnancy urine and seems to have similar chromatographic properties to aldosterone in the propylene glycol-toluene, Bush C, and Eberlein-Bongiovanni E<sub>2</sub>B systems. However, although these compounds of unknown identity may interfere with the assay under certain circumstances Neher and Wettstein (1960) report that of all the known compounds isolated from adrenals and those related to aldosterone prepared synthetically, only 17-isoaldosterone and 18-hydroxy compound A would be expected to be potential contaminants of aldosterone on the final chromatogram and these two

steroids have not yet been found in biological fluids in appreciable amounts. Under certain high pH conditions aldosterone is converted to 17-isoaldosterone. These two compounds are readily separated with the B5 solvent systems on partition columns and even, with care, in the corresponding paper system (Laumas and Gut, 1961).

### 2. Use of the Acetyl Derivative

It can be seen from Table II that the acetyl derivatives of the compounds described by Nowaczynski-Genest and also cortisol and cortisone monoacetate can be easily separated from aldosterone diacetate, and various methods therefore employ acetylation to gain specificity. Sobel *et al.* (1959) (Method R, Table IV), Brooks (1960) (Method S, Table IV) employ a two-paper separation of the free compound before the final paper chromatogram of the diacetate which is then measured by soda fluorimetry.

The method of Ayres *et al.* (1957a) (Method U, Table IV) employs two column separations of the free compound and diacetate then paper chromatography of the derivative. This method has been recently simplified by Flood *et al.* (1961) (Method T, Table IV) and now involves one high-resolution column separation of the free compound followed by paper chromatography of the diacetate and soda fluorimetry. Both these methods have high specificity and precision and the last version is suitable for multiple assays.

### 3. Use of Isotopic Indicators

Nearly all the previously described methods have the disadvantage that reproducibility and good recoveries are critically dependent on the skill of the operators. This can be avoided by the use of an isotope dilution technique where labeled hormone is added routinely as an automatic check on recoveries. The methods of Ayres *et al.* (1957a) (Method U, Table IV) and Kliman and Peterson (1960) (Method X, Table IV) do this by adding C<sup>14</sup>-aldosterone diacetate after the formation of the derivative, the recovery being corrected from this stage. Flood *et al.* (1961) (Method T, Table IV) add H<sup>3</sup>-aldosterone after neutral extraction and by this procedure, the recovery is fully corrected for provided that the steroid moiety in the 3-oxo conjugate is as sensitive to acid conditions as the free aldosterone.

### 4. Method of Kliman and Peterson (1960)

The general method of Kliman and Peterson (1960) (Method X, Table IV) employs, together with the C<sup>14</sup>-aldosterone diacetate indicator

technique as described, tritiated acetic anhydride to form the diacetate of the urinary aldosterone from the crude extract following hydrolysis at pH 1. The diacetate is fractionated on two-paper systems then another derivative, presumably 18-lactone 21-monoacetate, is formed by chromic acid oxidation and paper chromatographed. From the  $H^3/C^{14}$  ratio of this purified derivative, the amount of aldosterone present before acetylation can be calculated. This method has the advantage of great sensitivity (only 1/100th of a daily output of urine being required) and working with this small volume of aqueous phase, quantitative recovery of aldosterone to the stage of acetylation presents little difficulty. However, as with all labeled reagent methods (Avivi *et al.*, 1954) great care must be taken to check specificity and the  $H^3/C^{14}$  ratio must be constant on the final chromatogram. If more than a 1/50th daily output of urine must be taken then a further preliminary purification of the free aldosterone is recommended. The use of  $H^3$ -acetic anhydride is not expensive. However, if  $H^3$ -aldosterone were to be added to the urine to obtain a full recovery correction which seems desirable, the use of  $C^{14}$ -acetic anhydride would be necessary and commercial supplies of this reagent are much more costly. It appears that it would be advantageous for a supply of high specific activity  $C^{14}$ -aldosterone to be made generally available for use as indicator so that the economical tritiated acetic anhydride can be employed as the routine reagent for urinary work. The general method is easily capable of being applied for multiple assays and it seems that future versions will be used extensively in laboratories suitably equipped and financed.

#### C. GLUCURONIDE METABOLITES OF ALDOSTERONE

No complete details of any direct method for the estimation of tetrahydroaldosterone from human urine have yet been published. However, an indirect method for the calculation of its excretion has been reported (Flood *et al.*, 1961). Ulick *et al.* (1958), in their pioneering studies on this metabolite, also described a method for the measurement of its specific activity after the injection of tritiated aldosterone. Coppage *et al.* (1959) have given values for the excretion of the metabolite after administration of prednisone and SU4885 to normal subjects and have kindly supplied details of the method used. This method was developed in collaboration with Dr. S. Ulick.

##### 1. Method of Ulick *et al.* (1958)

Urine (an aliquot containing 5  $\mu$ g. or more metabolite) was incubated with  $\beta$ -glucuronidase Ketodase (200 units per milliliter of urine) for 3 days at 37°C. and pH 4.5 and extracted with ethyl acetate. The metabo-



lite was first separated on the paper system, ethylene dichloride-formamide which was run for 48 hours ( $R_{\text{tetrahydrocortisone}} = 0.7$ ). It was then eluted and acetylated with  $C^{14}$ -acetic anhydride, forming a triacetate. The acetyl derivative was chromatographed on the paper system, methylcyclohexane-formamide, which was run for 18 hours ( $R_{\text{DOCA}} = 1.05$ ). Final purification was achieved on a partition Celite column; stationary phase, methanol-water, 4:1; mobile phase, cyclohexane. The  $C^{14}/H^3$  ratios of the peak fractions were checked for uniformity and if found satisfactory, the value of the mean ratio allowed the initial tritium specific activity of the tetrahydroaldosterone to be calculated. The secretion rate was then also calculated from the radioactivity injected divided by this specific activity. Full details of this method are to be published.

### 2. Method of Coppage et al. (1959)

An aliquot of a 24-hour urine collection, containing more than 5 but less than 30  $\mu\text{g}$ . of the metabolite was initially extracted at neutral pH with methylene dichloride. The urine was then hydrolyzed with 75 units per milliliter of urine (Sigma  $\beta$ -glucuronidase) for 12 hours at pH 6.2 and 42°C. Fresh enzyme was added and the process repeated. The aqueous phase was extracted  $4 \times \frac{1}{2}$  volume ethyl acetate and the solvent washed with 0.2 N NaOH and then brought to neutrality with acetic acid and water. The extract was fractionated in the system formamide (50% formamide in methanol)-ethylene chloride for 18 to 26 hours at 30°C. ( $R_{\text{cortisol}}$  of metabolite = 0.78). Tetrahydroaldosterone was chromatographed for 14 hours on the Eberlein-Bongiovanni  $E_2B$  system ( $R_{\text{cortisol}} = 1.28$ ). After acetylation, the triacetate was run for 5 hours on washed paper in the modified Bush A system, ligroin-methanol-water (4:3:1 v/v/v), ( $R_{\text{DOCA}} = 1.07$ ). Following development of the paper with an alkaline solution of blue tetrazolium (0.03% in 1 N sodium hydroxide), the formazan was eluted with 10% HCl in pyridine and the density read at 560 m $\mu$ . An Allen type correction could be applied. The molar absorbance of DOCA and tetrahydroaldosterone triacetate were equivalent and hence the former was used as standard.  $C^{14}$ -Cortisol was used as an indicator for recovery losses on the first chromatogram and  $H^3$ -17-hydroxydeoxycorticosterone on the second. The daily excretion of tetrahydroaldosterone was some fourfold greater than the aldosterone released from the 3-oxo conjugate in the same urine. Full details of this method will also be published.

### 3. Method of Flood et al. (1961)

The secretion rate of aldosterone was estimated from the specific activity of the aldosterone released from the 3-oxo conjugate after an injection

tion of 7- $H^3$ -aldosterone. This part of the method was similar to that previously described (Section V, B, 2). An aliquot of urine (equivalent to a 2-hour collection) was first extracted at neutral pH with methylene dichloride and then incubated with 500 units per milliliter of urine (Keto-dase) at 37°C. for 24 hours and the aqueous phase extracted with ethyl acetate. After being washed, the extract was taken to dryness and chromatographed on a kieselguhr partition column, 60  $\times$  1 cm., 14-ml. stationary phase, 28 gm. Celite 545. The solvent system was water-methanol, 1:1, benzene-ethyl acetate, 15:1. The partition coefficients of tetrahydrocortisone, compound  $\alpha$  (tetrahydroaldosterone), and tetrahydrocortisol were 0.14, 0.09, and 0.07, respectively. From a knowledge of the quantity of tritium as compound  $\alpha$  and the secretion rate of aldosterone, the excretion of compound  $\alpha$  per day in micrograms could be calculated. The amount of compound  $\alpha$  (tetrahydroaldosterone) (both as per cent tritiated aldosterone injected and micrograms excreted) in the urine of normal subjects seems to be rather higher as measured by the method of Flood *et al.* (1961) compared with the values obtained by Ulick and Liddle, Island, and Coppage (1961). However, as previously mentioned, Liddle and co-workers find the same percentage of an injected dose of tritiated aldosterone as tetrahydroaldosterone as Flood *et al.* (1961) report for compound  $\alpha$  and hence the differences are more likely to be due to variation in secretion rates between the two groups of patients. On the other hand, Melby (1960), using a method which measures the metabolite by the Lewbart and Mattox (1961) reaction after chromatography of the free compound only, reports values which are much higher. In the light of the recent findings of Ulick and Kusch (1961), it may be that Melby is measuring a mixture of tetrahydroaldosterone and 3 $\alpha$ ,18,21-trihydroxy-5 $\beta$ -pregnane-11,20-dione, which are difficult to separate unless the acetyl derivatives are formed.

#### D. ASSAY OF FREE ALDOSTERONE IN URINE

It has been suggested that the amount of free cortisol in urine will reflect the mean value in blood during the time of the urinary collection of the free steroid (Greaves and West, 1960) but in pregnancy the urinary cortisol values are increased (K. M. Jones *et al.*, 1959) whereas it is probable that the unbound plasma concentrations are not raised to the same extent. However, aldosterone is only very weakly bound to plasma proteins (Mills and Bartter, 1959) and the difficulties of measuring plasma concentrations of this hormone are so great that it may be worthwhile to explore the validity of measuring the urinary free aldosterone as an indication of mean blood values.

1. *Method of K. M. Jones et al. (1959)*

After extraction of a whole daily output of urine at neutral pH by chloroform and silica gel chromatography, 0.1  $\mu\text{g}$ .  $\text{H}^3$ -aldosterone was added to the eluate (routinely this could be added to the urine). The extract was then chromatographed on a partition column 60 cm. long using 28 gm. Celite 545, 14-ml. stationary phase (methanol-water, 1:1 v/v) with benzene as the mobile phase. The fractions containing most tritium were pooled and acetylated and chromatographed on a further column, 30 cm. long, using 14 gm. kieselguhr, 7-ml. stationary phase (methanol-water, 4:1 v/v) with benzene-petroleum ether (2:3 v/v) as the mobile phase. The fractions containing tritium were run as one spot against three dilutions of standard aldosterone diacetate in the Bush B3 system and the steroid measured by soda fluorimetry. From the specific activity of the final spot, the amount of free aldosterone (corrected from the stage of addition of indicator) could be calculated. The specificity of the method was checked by scanning for tritium and soda fluorescence on the final chromatogram (Tait and Tait, 1960). Good correlation between these two measurements was observed.

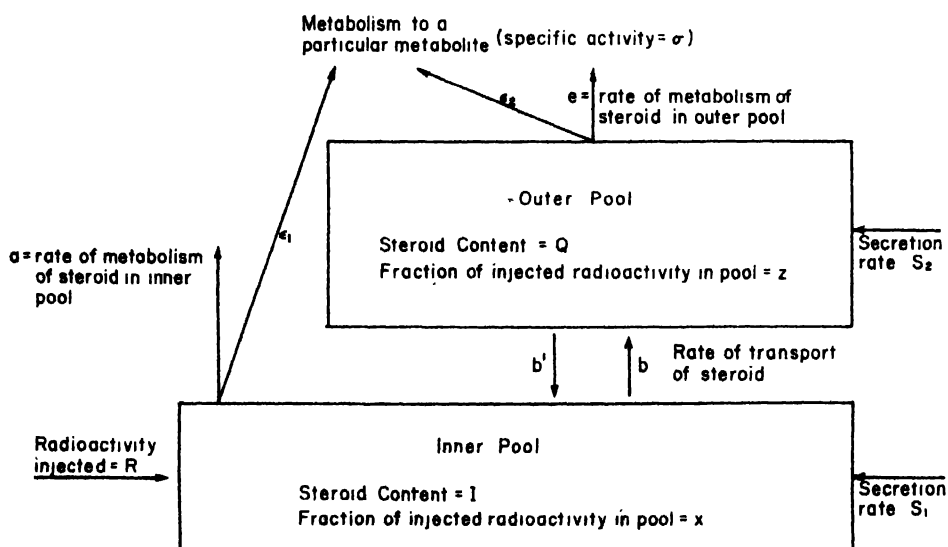
The values of urinary free aldosterone in six normal women were 0.17–0.63, mean value 0.44  $\mu\text{g}$ ./24 hours (0.19–0.43, mean value 0.24% dose injected). Corresponding values in six pregnant women were 0.7–3.5, mean value 1.7  $\mu\text{g}$ ./24 hours (0.22–0.33, mean value 0.29% of the dose injected). If the urinary free aldosterone reflects the mean blood concentration, these results indicate that although the secretion rate and pattern of metabolism change greatly in pregnancy, the turnover rate of the hormone remains nearly constant. Recent studies (Tait *et al.*, 1961a) on the plasma disappearance curve of radioactive aldosterone (Section VII, B, 1) in pregnant women confirm this conclusion. This approach of measuring the urinary free aldosterone to indicate plasma concentrations would not be hopeful if the 3-oxo conjugate were hydrolyzed to release free aldosterone in the bladder or urine before the neutral extraction was carried out. However, in the investigations on pregnancy urine (K. M. Jones *et al.*, 1959) there was no correlation of the free aldosterone with the 3-oxo conjugate although the proportion of the 3-oxo conjugate in the urine to the secretion rate varied widely. This indicates that the method is unlikely to be invalid for this reason, although further investigations on a variety of clinical conditions will be necessary, before it gains acceptance on general grounds. The method of Kliman and Peterson (1960) and Flood *et al.* (1961), would appear to be also conveniently applicable for the measurement of this parameter,

although greater specificity is required than for the estimation of the aldosterone released from the 3-oxo conjugate.

## VI. Indirect Secretion Rate Estimates in Man

### A. ASSUMPTIONS

This method depends on the over-all assumption that because a certain proportion of a single injection of a radioactive hormone is excreted as a particular metabolite then exactly the same proportion of the endogenous secretion will appear in the urine as this metabolite. If this is true then the total secretion equals radioactivity injected divided by the specific activity of the metabolite ( $S = R/\sigma$ ). This expression is correct only if certain particular assumptions are valid and appropriate precautions taken.



Total secretion =  $(S_1 + S_2)T = S$ ;  $\sigma$  = specific activity of metabolite in time  $T$

$$\frac{R}{\sigma} = S \left[ \frac{1 + \left( \frac{a}{a+e} \right) \left( \frac{S_2}{b} \right)}{1 + \left( \frac{a}{a+e} \right) \left( \frac{S_2}{b} \right)} \right]$$

Fig. 3. Model for consideration of secretion rate methods.

Thus, if the two-compartmental model as seems to be appropriate to describe the metabolism and transport of aldosterone in man, Tait *et al.*, 1961b), described in Fig. 3, is considered

$x$  = fraction of injected radioactivity in inner pool

$z$  = fraction of injected radioactivity in outer pool

$I$  = steroid content in inner pool

$Q$  = steroid content in outer pool

$b$  = rate of transfer of steroid from inner to outer pool

$b^1$  = rate of transfer of steroid from outer to inner pool

$a$  = rate of metabolism of steroid in inner pool

$e$  = rate of metabolism of steroid in outer pool

$S_1$  = rate of secretion of steroid into inner pool

$S_2$  = rate of secretion of steroid into outer pool

$S$  = total secretion of steroid =  $(S_1 + S_2)T$  in time  $T$

$R$  = radioactivity injected into inner pool

and then

$$\frac{dx}{dt} = \frac{-ax}{I} - \frac{bx}{I} + \frac{b^1z}{Q} \quad (1)$$

$$\frac{dz}{dt} = \frac{-cz}{Q} - \frac{b^1z}{Q} + \frac{bx}{I} \quad (2)$$

In the steady state  $e = S_2 + b - b^1$  and if  $\epsilon_1$  = rate of excretion (combination of rate of formation and renal clearance) of a particular metabolite from the inner pool,  $\epsilon_2$  = rate of excretion of same metabolite from outer pool and  $\sigma$  = specific activity of this metabolite in time  $T$  (when all radioactivity in the form of the metabolite has been excreted), it can be shown by treatment similar to that used by Laumas *et al.* (1961a), Laumas *et al.* (1961b), that

$$R = S \frac{1 + [a/(a + e)](S_2/b)}{1 + [\epsilon_1/(\epsilon_1 + \epsilon_2)](S_2/b)}$$

If  $e = 0$  (or  $\epsilon_2 = 0$ ) or  $S_2 = 0$ , then  $R/\sigma = S$ .

Therefore unless both secretion and metabolism occur in a compartment into which the radioactive hormone has not been injected, the expression will be correct.

This proves rigorously that the calculation is likely to be correct on theoretical grounds except in rather particular clinical situations, e.g., in pregnancy when the radioactive steroid is injected in the maternal blood and the fetus secretes and metabolizes the hormone. However, this treatment shows that it is quite inappropriate to apply the term "isotope dilution" generally to this method.

In addition the following more technical conditions must be met.

1. The amount of steroid injected must not be great enough to alter the normal metabolism and transport of the hormone. As 7- $H^3$ - $d$ -aldosterone of high specific activity (20  $\mu$ c. per microgram) is now generally

available, this assumption should be easily satisfied in the future. An injection of 0.1  $\mu\text{g.}$  of aldosterone at 20  $\mu\text{c.}$  per microgram is sufficient for the routine application of the method and it is possible to use 0.02  $\mu\text{g.}$  and still obtain reasonable radioactivity in the urinary metabolites.

2. The injected compound should be in the same chemical form as the secreted hormone. This assumption requires careful continuous checking of the radiochemical purity of the material to be injected. In particular, it is essential to administer the natural optical antipode of the hormone (*d*-aldosterone) as Ulick (1961) has shown that 7- $\text{H}^3$ -aldosterone is metabolized neither to the urinary 3-oxo conjugate nor tetrahydroaldosterone. Secretion rate estimates after injection of tritiated racemic mixture, which was used in some earlier studies, will therefore be too high by a factor of two.

3. The radioactive isotope must be stably attached to the injected hormone and the heavier atom in the molecule must not affect its behavior in the body. The metabolism of 7- $\text{H}^3$ - and 16- $\text{H}^3$ -aldosterone (Flood *et al.*, 1961; K. M. Jones *et al.*, 1959) are similar. Also Peterson (1959) reported that the metabolism of randomly labeled  $\text{H}^3$ -corticosterone and 4- $\text{C}^{14}$ -corticosterone are identical. Therefore, it seems that when only one atom is replaced in a fairly large molecule, such as a steroid, this assumption will be valid for clinical applications.

4. The pattern of metabolites produced, the volumes of distribution of the hormone and metabolites, and the renal clearance of the metabolites should be constant during the time of the urine collection. However, it seems probable that the secretion rate may vary during this time if the other parameters remain constant and the estimate would still give a valid mean value.

5. The metabolite measured must be derived exclusively from the hormone. In view of the rather unique nature of the metabolites, the 3-oxo conjugate and tetrahydroaldosterone, it seems highly likely that this assumption is valid for aldosterone secretion rate estimates.

6. All (>95%) the radioactivity in the form of the metabolite must be collected. The studies of Laragh *et al.* (1960) and Flood *et al.* (1961) indicate that for subjects with normal renal function this assumption is satisfied for a 24-hour urine collection when the specific activity of the aldosterone from the 3-oxo conjugate is measured but a 48-hour collection must be made for tetrahydroaldosterone. Laragh *et al.* (1960) have reported that for subjects with malignant hypertension and subnormal renal function, only 75% of the radioactivity in the form of tetrahydroaldosterone is excreted in the first 24 hours after injection. In one terminal case less than 50% was excreted in the same time. No corresponding studies have been made with 3-oxo conjugate but it is

to be expected that excretion will be much quicker for this metabolite even in cases of renal dysfunction. After treatment with Enovid about 15% of the radioactivity in the form of tetrahydroaldosterone is excreted in the second 24 hours whereas the 3-oxo conjugate radioactivity is entirely excreted by this time (Layne, 1961). From these considerations, a measurement of the specific activity of the 3-oxo conjugate rather than that of tetrahydroaldosterone would seem preferable. However, when the secretion rate varies markedly and the mean value for more than 24 hours is required, e.g., as in the diagnosis of Conn's syndrome, it is theoretically possible that a more valid estimate will be obtained by measuring the specific activity of tetrahydroaldosterone. This is because any variation in the rate of production and renal clearance of the metabolite during this time will be reflected to some extent in altered excretion of radioactive as well as nonisotopic tetrahydroaldosterone.

Nearly all these assumptions can be tested by comparing the specific activities of various metabolites. If these are equal this indicates that the assumptions are probably valid (Laumas *et al.*, 1961). Such an investigation has so far been carried out in normal subjects only (Laumas *et al.*, 1961). Ulick *et al.* (1958) have tested the general validity of the method by infusing *dl*-aldosterone into an adrenalectomized subject and giving a single injection of tritiated *dl*-aldosterone. Rosenberg (1961) has administered 10 mg. of *d*-aldosterone to a normal subject for 10 days and gave a single injection of 7- $H^3$ -*d*-aldosterone after 4 days. In both cases the calculated "secretion rate" agreed with the rate of administration of exogenous steroid.

One potential disadvantage of the radioactive method lies in the limitation on repeated measurements imposed by rules of radiation hygiene and the necessity to allow for complete excretion of radioactivity in the form of a particular metabolite. However, secretion rates can readily be determined after the injection of 0.4  $\mu$ c.  $H^3$ -aldosterone and this would allow for five serial determinations. In most subjects, a 48-hour collection of urine would suffice particularly if the 3-oxo conjugate were studied. For many clinical investigations these limitations would not be serious.

## B. METHODS

After an injection of radioactive aldosterone, about 75% of the radioactivity in the crude extract obtained after hydrolysis of the urine at pH 1 is in the form of the hormone itself. Also, about the same proportion of the radioactivity in the total extract made after incubation of the urine with  $\beta$ -glucuronidase, is present as tetrahydroaldosterone. This

means that the isolation of radioactivity specifically in the form of the two metabolites presents little difficulty and most of the methods described for the measurement of tetrahydroaldosterone or the aldosterone released from the 3-oxo conjugate can be readily adapted for the estimation of their specific activity. This determination is actually less demanding than the measurement of the amount of metabolite in urine as quantitative or known recovery is not required. The following methods have been described:

### 1. *Method of Ulick et al. (1958)*

This method for the estimation of secretion rates from the specific activity of tetrahydroaldosterone has been described previously (Section V, C, 1). The specific activity of the metabolite in a 24-hour collection of urine after the injection of randomly labeled  $H^3$ -*d*-aldosterone is measured. Values for normal subjects on an unselected diet were found to be 150–330 (8 subjects), mean 200  $\mu$ g. per day (Laragh *et al.*, 1960). These estimates are about 8% too high because of the incomplete collection of the radioactivity in the form of tetrahydroaldosterone. More recent determinations using this method (Laragh, 1960) have given lower values for normal secretion rates for reasons which are not yet clear.

This method has been applied to the study of primary hypertension, unilateral renal disease, primary aldosteronism, and malignant hypertension (Laragh *et al.*, 1960).

### 2. *Method of K. M. Jones et al. (1959)*

This method is identical with that of Flood *et al.* (1961) (Table IV, Method T) for the estimation of the specific activity of the aldosterone released from the urinary 3-oxo conjugate except in a few practical details. Normal and pregnant women have been investigated after the injection of 16- $H^3$ -aldosterone. The secretion rates of six normal women on an unselected diet were found to be 72–315, mean 192  $\mu$ g. per day and that of four normal men, 86–116, mean 96  $\mu$ g. per day.

In the early studies of Ayres *et al.* (1957b), who first used such a method, the secretion rates of two normal men on a normal diet were 170 and 190  $\mu$ g. per day and there was a fourfold increase on a low salt diet.

### 3. *Method of Flood et al. (1961)*

This method (Table IV, Method T) again measures the specific activity of the aldosterone after injection of 7- $H^3$ -steroid. It is identical with the procedure of estimating the aldosterone released from the 3-oxo conjugate except, of course, labeled hormone need not be added to the



urine. The secretion rates of five normal women was found to be 50–118, mean value 77  $\mu\text{g.}$  per day. In a study on another group of five normal women, the values were 45–255, means 128  $\mu\text{g.}$  per day (Tait *et al.*, 1961b).

#### 4. Method of Peterson (1959)

The method of Kliman and Peterson (1960) (Method X, Table IV) can be readily adapted to the measurement of the specific activity of the aldosterone from the 3-oxo conjugate. Here  $\text{C}^{14}$ -acetic anhydride must be used as the labeled reagent after injection of tritiated aldosterone. This modification has not yet been published in detail but probably requires little alteration in methodology. It has also been used by Muller and Manning (1960). Secretion rates for three normal subjects on unselected diets were found to be 350, 330, and 400  $\mu\text{g.}$  per day. As  $\text{H}^3$ -*dl*-aldosterone was used in these studies, these values should be halved to give the correct secretion rates (Ulick, 1961). Recent results of Peterson (1960), using the same method but injecting 7- $\text{H}^3$ -aldosterone, give much lower values for normal secretion rates (about 100  $\mu\text{g.}$  per day).

It is perhaps to be expected that there will be significant differences in normal values between different groups even when the nature and purity of the injected radioactive hormones is satisfactory. The differences in the values found by Flood *et al.* (1961) and K. M. Jones *et al.* (1959) using similar methods for normal women are not so much in the proportion of radioactivity excreted as the metabolite but in the amount (micrograms) of aldosterone released from the 3-oxo conjugate. Hence it is likely that the variation in normal secretion rates lies in differences of diet and activities of the subjects and these must be carefully controlled in most investigations if meaningful comparisons of secretion rates are to be made.

### VII. Assay in Peripheral Blood

Correlation of the biological effects of a hormone with assays of its concentration in the body requires specifically a knowledge of its concentration at the target organ. The nearest practical approach to such an assay in man lies in the measurement of its concentration in peripheral plasma. For cortisol and thyroxine, it is considered by many investigators that the appropriate assay is of the "unbound" concentration in plasma, i.e., the amount of hormone which is not bound to proteins other than albumin (Mills and Bartter, 1959; Ingbar and Freinkel, 1960).

## A. DIRECT MEASUREMENTS

The measurement of the amount of aldosterone in peripheral plasma is extremely demanding both as regards sensitivity and specificity. Early studies on large quantities of plasma, bioassayed following column chromatography, showed that the concentration in normal subjects was of the order of  $0.03 \mu\text{g./100 ml. plasma}$ . This level requires that a suitable assay method must be able to measure about  $1 \mu\text{g.}$  of hormone and labeled reagents seem to be necessary to achieve this order of sensitivity. However, as previously discussed in detail, such methods have low intrinsic specificity. Earlier results, using both labeled acetic anhydride (Peterson, 1959) and pipsyl reagent (Bojesen, 1958) have been found to be too high due to this reason.

*1. Method of Bojesen and Degn (1960) (Method Y, Table IV)*

Plasma (20–30 ml.) was extracted with chloroform and the dried residue esterified with  $\text{S}^{35}\text{-}p\text{-iodophenylsulfonic anhydride}$  (20–150 mc./millimole). Aldosterone  $\text{I}^{131}\text{-}p\text{-iodophenylsulfonate}$  was then added as indicator. The washed extract was then chromatographed on the paper system ethyl oleate-acetic acid-water followed by the formamide-chloroform system. The steroid was then oxidized with chromic acid and the product rechromatographed on the formamide system. The  $\text{S}^{35}/\text{I}^{131}$  ratio of the final spot was tested for uniformity and if satisfactory, the value allowed the calculation of the aldosterone present before esterification. Recovery, to this stage, was reproducibly 80%.

Concentrations in the peripheral plasma of dogs on a normal salt diet were found to be  $0.01\text{--}0.03 \mu\text{g./100 ml. plasma}$ . After adrenalectomy, the value was  $<0.01 \mu\text{g.}\%$ . Determinations in human peripheral plasma of three normal subjects gave  $<20, 25, \text{ and } 16 \text{ m}\mu\text{g./100 ml. plasma}$  (Bojesen, 1960). Earlier results gave much higher values due to  $\text{S}^{35}$  in the final extract not derived from aldosterone (Bojesen, 1958). One disadvantage of this method is that the pipsyl derivative of aldosterone is unstable and chromatography must be carried out in the cold (Bojesen, 1960).

*2. Method of Peterson (1959)*

The method of Kliman and Peterson (1960) (Table IV, Method X) and Davis *et al.* (1958) (Table IV, Method W) uses tritiated acetic anhydride (about  $150 \text{ mc./mM}$ ) as reagent and  $\text{C}^{14}\text{-aldosterone diacetate}$  as indicator.

Preliminary results indicated that normal human plasma concentra-

tions were 0.04–0.08  $\mu\text{g./100 ml.}$  plasma (Peterson, 1959). These estimates were undoubtedly too high. Bojesen (1961) suggests that this may be due to trace amounts of free aldosterone in the indicator which react with the  $\text{H}^3$ -acetic anhydride. Later investigations showed that lower values were more appropriate (about 0.01  $\mu\text{g./100 ml.}$ ) (Peterson, 1960). These recent estimates have presumably required modifications of the original method and publication of these are awaited with interest.

### B. INDIRECT MEASUREMENTS

The concentration of aldosterone in peripheral blood is a result of a combination of its secretion and a quantity (secretion rate/plasma concentration) which has previously been termed a turnover rate (Tait *et al.*, 1961b) but which should be more appropriately—the metabolic clearance rate. The clearance rate will depend on the volumes of distribution and rate of metabolism of aldosterone. The secretion rate can be determined by any of the methods already discussed and the clearance rate can be estimated concomitantly either after a single injection or continuous infusion of labeled hormone by following the plasma radioactivity measured specifically as aldosterone.

#### 1. Method of Tait *et al.* (1961b): Single-Injection Method

Two microcuries of 7- $\text{H}^3$  aldosterone was given as a single injection. Plasma was withdrawn at 7.5, 15, 22.5, 30, 50, and 70 minutes after injection and  $\text{C}^{14}$ -aldosterone was added to every sample. The plasma was extracted with chloroform and after the solvent was taken to dryness the residue was acetylated. Aldosterone diacetate was then chromatographed on the kieselguhr partition column,  $60 \times 1 \text{ cm.}$ , 28 gm. Celite 545; 14-ml. stationary phase: methanol-water (4:1 v/v); mobile phase: toluene-Skellysolve C (2:3, v/v). The  $\text{H}^3/\text{C}^{14}$  ratio of the peak fractions, which were constant, allowed the calculation of tritium initially present as aldosterone. The plasma disappearance curve could be represented by the equation  $x = Ae^{-\alpha t} + Be^{-\beta t}$  where  $x$  is the percentage of the dose injected per liter plasma at time  $t$  and  $A$ ,  $B$ ,  $\alpha$ , and  $\beta$  are constants. On using a two-compartmental model, the clearance rate could be calculated as  $M_2 = \alpha\beta/A\beta + B\alpha$ . This equation depends on their being no metabolism ( $e$ ) or secretion ( $s_2$ ) in the outer compartment (Fig. 3).

#### 2. Method of Tait *et al.* (1961b): Constant Infusion Method

A single injection of 1  $\mu\text{c.}$  of 7- $\text{H}^3$  aldosterone was given and then after 30 minutes, 1  $\mu\text{c.}$  of tritiated aldosterone was infused over a period of 90 minutes. Plasma was taken 15, 30, 105, and 120 minutes after the

single injection and the tritium as aldosterone determined as in the previous method. If the radioactive concentration was constant at the end of the infusion (105 and 120 minutes after injection), the rate of radioactive infusion divided by the final concentration of radioactive aldosterone equaled the turnover rate. The secretion rate was again determined concomitantly by the method of Flood *et al.* (1961). A clearance rate estimation on one normal gave a value in agreement with the previous method. It is possible that this procedure, which in its simplest form requires two measurements of plasma radioactivity only, could be a convenient method of determining plasma concentrations of aldosterone.

It should be noted that the estimates obtained by the methods of group B gives mean values during the whole 24 hours whereas those of group A are estimates at a particular time and so, due to diurnal variation, these two sets of results may not agree.

In addition there may be discrepancies between the two types of method for more fundamental reasons:

In the model represented by Fig. 3, if  $r$  = rate of radioactive infusion into inner compartment and  $x$  and  $z$  = radioactivity in inner and outer compartments, respectively, then

$$\frac{dx}{dt} = 0 \text{ at end of infusion}$$

Therefore

$$r - \frac{ax}{I} - \frac{bx}{I} + \frac{b^1z}{Q} = 0$$

Also

$$\frac{dz}{dt} = 0 = \frac{-cz}{Q} - \frac{b^1z}{Q} + \frac{bx}{I}$$

Then again in the steady state

$$c = b - b^1 + S_2$$

Then it can be shown that

$$I \cdot \frac{r}{x} = a + \frac{cb}{S_2 + b}$$

i.e.,

$$\frac{r}{x} = \frac{a + e}{I} \left[ 1 - \frac{S_2 e}{(S_2 + b)(a + e)} \right]$$

This leads to the expression

$$\left[ \frac{r}{\text{plasma concentration of radioactivity}} \right] = \left[ \frac{\text{secretion rate}}{\text{plasma concentration of steroid}} \right] \left[ 1 - \frac{S_2 e}{(S_2 + b)(a + e)} \right]$$

Provided that  $S_2 = 0$ , the expression used in the infusion method, B2 is therefore correct even if metabolism occurs in the outer compartment and it can be proved that this is also the case for the calculation of metabolic clearance rate by method B1. If both  $S_2$  and  $e \neq 0$ , i.e., both secretion and metabolism occur in the outer compartment, neither the single injection nor the continuous infusion calculation will give the correct clearance rate or peripheral plasma concentration.

### C. PLASMA PROTEIN BINDING OF ALDOSTERONE

Studies on cortisol indicate that the effective concentration of the hormone is not the total value in peripheral plasma but the unbound amount. Hence, for many purposes it is necessary to estimate this parameter. The studies of Mills and Bartter (1959) Daughaday *et al.* (1961), Sandberg *et al.* (1960) and Meyer *et al.* (1961) indicate that aldosterone is only weakly bound to plasma proteins and even in pregnancy the significant but small binding to proteins other than albumin is not appreciable (Meyer *et al.*, 1961; Daughaday *et al.*, 1961). Therefore it seems that this is not an important factor in determining the effective concentration of aldosterone in most clinical conditions. However, after treating women with large amounts of estrogens (0.3 mg. ethynyl estradiol 3-methyl ether daily), the binding of aldosterone increases appreciably and this may be physiologically significant (Layne, 1961).

The binding of aldosterone may be determined by an ultrafiltration (Mills and Bartter, 1959) or microdialysis equilibrium technique (Meyer *et al.*, 1961). Both are suitable for the examination of small quantities of human plasma.

## VIII. Assays of the Adrenal Gland

### A. ADRENAL GLAND AND INCUBATE ANALYSIS

The analysis of adrenal tissue, before or after incubation, is not particularly demanding as regards specificity compared with the assay of other biological materials for aldosterone. Neher and Wettstein (1960) report that no known compound from adrenal glands will interfere in the assay of the hormone by their method if it is carried out precisely. Therefore any of the methods described in Tables III and IV are suitable for this type of analysis.

Assay of human adrenal tumors (Neher, 1958; Conn, 1961) is the major application of these methods when the tissue is not incubated.

Neher (1958) gives the following procedure: Tissue, obtained not longer than 1-2 hours after death, was chopped and homogenized in saline. The suspension was poured into 10 to 20 times the volume of 80% acetone and stirred for several hours. The residue was filtered and washed with 80% acetone. The total aqueous acetone mixture was concentrated *in vacuo* and the resulting aqueous phase extracted with chloroform. The chloroform was then concentrated and diluted with petroleum ether to a 1% mixture and this was extracted four times with half-volume 60% methanol saturated with sodium chloride. The aqueous methanol was then concentrated and extracted with chloroform. This extract was then analyzed by the method of Neher and Wettstein (1956) (Table III, Method G) including chromatography on silica gel column.

After incubation, in most methods the fluid is extracted with chloroform. Generally this is a clean extract and needs little further purification before chromatography. Ayres *et al.* (1960) found that about 80% of the total aldosterone at the end of incubation of ox adrenals was extracted by this method and only small amounts remained in the tissue. This is in contrast to corticosterone, where 50% of this steroid was present in the tissue and was not extracted by this procedure. The usual soda fluorescence methods are of adequate sensitivity for the assay of incubation fluid from ox adrenals. Analysis of rat adrenals, after incubation by the method of Safran and Schally (1955) requires greater sensitivity. Bioassay is used in certain procedures (Table III, Method F) (Stachenko and Giroud, 1959).

Studies on the biosynthesis of aldosterone using radioactive substrates have required a more elaborate method for the determination of the final specific activity of the hormone (Table IV, Method V) (Ayres *et al.*, 1960). The problem of this type of measurement, in contrast to the determination of the specific activities of metabolites in urine, lies in the elimination of radioactive impurities rather than in any difficulty in the assay of nonisotopic aldosterone. Some of these impurities, which may be intermediates in the biosynthesis of aldosterone, e.g., 18-hydroxy-corticosterone, are difficult to remove as they tend to streak on partition chromatography. Neher and Wettstein (1960) have reported that, due to tautomerization, this is a property of certain 18-hydroxy steroids. As expected, if these compounds are intermediates with an appreciable turnover rate, preincubation and longer times of incubation may reduce this problem (Giroud and Stachenko, 1960).

## B. ADRENAL VENOUS BLOOD

Investigations of the mode of control of aldosterone secretion have depended to a considerable extent on the assay of dog, sheep, or rat adrenal

venous blood. Sensitivity of the assay is a very important factor in such investigations. The estimated output of these animals on normal electrolyte balance is of the order of 25  $\mu\text{g.}$  per hour per 100 kg. body weight per gland (Farrell, 1958), when large amounts of blood are taken for analysis from the dog and about 5  $\mu\text{g.}$  per hour per 100 kg. body weight per gland (Davis *et al.*, 1958), when smaller volumes are analyzed. This latter amount is in agreement with the *in vivo* estimates in man by the urinary specific activity method. In the cat (Newman *et al.*, 1958) and the rat (Singer and Stack-Dunne, 1955) the outputs, after bleeding, are about 50  $\mu\text{g.}$  per hour per 100 kg. body weight per gland. Denton (1960) has estimated that in adrenal transplants in sodium replete sheep, the output of aldosterone is about 0.6–1.8  $\mu\text{g.}$  per hour per 100 kg. body weight per gland. If the adrenal blood flow is 14 ml. per minute (unrestricted venous output) and the hematocrit 25%, plasma flow will be 630 ml. per hour. If 50 ml. plasma is extracted under these physiological conditions, about 0.08  $\mu\text{g.}$  aldosterone will be available for assay. This is reduced even further when the animal is on a high salt diet, or after hypophysectomy, or under certain conditions of decerebration and this has prevented extensive studies on the inhibitory aspects of the mode of control of the hormone. Also, if large amounts of blood are taken for the assay to compensate for the lack of sensitivity of the method, the secretion of aldosterone will be stimulated by the analytical process.

In the early experiments on the detection of aldosterone in adrenal venous blood, the hormone was bioassayed after a single chromatographic run (Simpson *et al.*, 1952) or detected after chromatography and hydrolysis of the diacetate (Simpson *et al.*, 1952; Farrell *et al.*, 1954). The first type of method is still employed (Ganong *et al.*, 1959; Mulrow *et al.*, 1959; Method A, Table III) (Das Gupta and Giroud, 1959; Method B, Table III) (Singer and Stack-Dunne, 1955; Method C, Table III) and is capable of measuring 0.4  $\mu\text{g.}$  aldosterone (Table I). The second type of method has been refined by Farrell *et al.* (1954) (Method P, Table IV) and the hormone is detected by bioassay or ultraviolet absorption with sensitivity of about 2  $\mu\text{g.}$  The other methods employed use two or three chromatographic separations of the free compound before detection by bioassay (Stachenko and Giroud, 1959; Method F, Table III, sensitivity 0.4  $\mu\text{g.}$ ) or by soda fluorescence which is usually checked by other physicochemical methods (Neher and Wettstein, 1956; Singer, 1960, Method G, E, Table III; Stachenko and Giroud, 1959, Method F, Table III; Reich, 1958, Method J, Table III; Bartter *et al.*, 1960, Method K, Table III). In methods G, E, F, and J the fluorescence is examined visually whereas in methods E of Singer and K it is measured by a fluorimeter. The sensitivity is generally about 2  $\mu\text{g.}$  How-

ever, Bartter *et al.* (1960) have improved this by concentrating the steroid in a small area and claim a sensitivity of 0.2  $\mu\text{g}$ .

The method of Davis *et al.* (1958) (Method V, Table IV) used the labeled reagent assay of Kliman and Peterson (1960) after extraction of the plasma with methylene dichloride. As described previously, tritiated acetic anhydride was used as the reagent and  $\text{C}^{14}$ -aldosterone diacetate as the indicator. The sensitivity was of the order of 0.01  $\mu\text{g}$ . and the final  $\text{H}^3/\text{C}^{14}$  ratio was satisfactorily uniform and constant. In the routine form of the method the recovery was automatically corrected after addition of the indicator, i.e., only after acetylation. However, when  $\text{H}^3$ -aldosterone was added to the plasma and  $\text{C}^{14}$ -acetic anhydride was used for acetylation, similar results were obtained (Kliman and Peterson, 1960) although the recovery was then automatically corrected throughout the procedure. In order to avoid errors caused by occasional poor recoveries due to damp acetic anhydride, large amounts of impurities in the extract, or other possible reasons it would appear that the use of the double isotope dilution derivative method is better. The present ready availability of  $\text{H}^3$ -aldosterone should enable this procedure to be adapted as the routine method and although the use of  $\text{C}^{14}$ -acetic anhydride is almost prohibitively expensive for multiple urinary analyses, this is less of a disadvantage in adrenal venous blood assays. Nevertheless as with the urinary method a supply of  $\text{C}^{14}$ -aldosterone would be advantageous at the present time to allow the use of the cheaper  $\text{H}^3$ -acetic anhydride in some experiments.

The method of Bojesen and Degn (1960) (Method Y, Table IV) using  $\text{S}^{35}$ -pipsyl reagent and aldosterone  $\text{I}^{131}$ -pipsan as the indicator has also been applied to the analysis of dog adrenal plasma with encouraging results. The sensitivity was about 0.002  $\mu\text{g}$ . Again it should be emphasized that when estimating such small quantities, uniformity of the isotope ratio in the final product is not the only criterion that should be used for specificity. Due to possible reactions of the reagent with trace amounts of free steroid in the added indicator, a blank value should always be established. As for the routine method of Kliman and Peterson (1960) recovery was only corrected after the esterification step. This disadvantage could also be overcome by the addition of  $\text{H}^3$ -aldosterone to the plasma and the use of  $\text{S}^{35}$ -pipsyl reagent.  $\text{S}^{35}$ -pipsyl reagent of the appropriate specific activity will be much cheaper than  $\text{C}^{14}$ -acetic anhydride and this should more than compensate for the 3-month half-life of  $\text{S}^{35}$ .

The complexity and expense of labeled reagent methods may be disadvantageous compared with other simpler and satisfactory methods for the urinary assay of the aldosterone from the 3-oxo conjugate but these



are minor factors relative to the labor involved in the whole experiment in the analysis of adrenal venous blood. Therefore, in view of the prime importance of achieving sensitivity which, as already discussed, should ideally be better than 0.08  $\mu\text{g}$ . it seems that labeled reagent methods will become the preferred procedure for this type of analysis unless entirely new methods with the required sensitivity and specificity are developed, e.g., phosphorimetry (Keirs *et al.*, 1957). On the other hand, the speed and resolution of gas chromatographic purification may enhance the advantages of labeled reagent methods if suitable derivatives are found.

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## Chapter 8

# Adrenaline and Noradrenaline

FRED ELMADJIAN

I. Introduction . . . . .	337
II. Chemistry . . . . .	337
A. Lutines . . . . .	338
B. Ethylenediamine Conjugation . . . . .	339
III. Colorimetric Methods . . . . .	341
A. Iodine Method . . . . .	342
B. Permanganate Method . . . . .	342
C. Arsenomolybdic Acid Method . . . . .	343
D. Naphthoquinone Condensation . . . . .	343
IV. Fluorometric Methods . . . . .	344
A. Trihydroxyindole Method . . . . .	344
B. Ethylenediamine Conjugation . . . . .	345
References . . . . .	347

## I. Introduction

Measurement of adrenaline (A) and noradrenaline (NA) may be achieved by colorimetric methods, bioassay, and, in the past decade, by fluorometric methods which have come to prominence. Bioassay methods and procedures for the extraction and separation of A and NA have been discussed in Volume I of this series. This chapter will deal with the chemistry and colorimetric and fluorometric methods for the determination of A and NA.

## II. Chemistry

Adrenaline (Fig. 1, I) and noradrenaline (Fig. 1, II) are catechol  $\beta$ -ethanolamines and, therefore, have chemical properties of phenols, alcohols, and amines. Reactions with oxidizing agents, reducing agents, and metals, as well as conjugation, are possible. Complete loss of biological activity is achieved with alkaline pH and heat. The racemic form may result from overheating even in acid solution, which causes loss of biological activity. Ascorbic acid is used quite extensively as a preserving agent during chemical manipulation. The use of solvents

presents difficult problems because these amines are highly soluble in aqueous media and solvents which are miscible with water, such as alcohol and acetone. Redistillation of solvents is essential, since traces

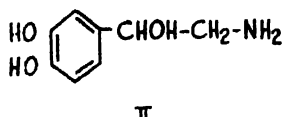
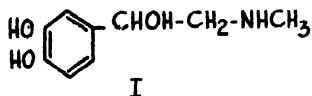


FIG. 1. Adrenaline(I) and noradrenaline(II).

of impurities will cause loss of biological activity. Glassware used should receive special attention during cleaning. Optical density measurements of catechol amines at  $279\text{ m}\mu$  are useful for general purposes, though not specific for A and NA. Though catechols as a group have a natural fluorescence which is activated at  $285\text{ m}\mu$  and fluoresces at  $325\text{ m}\mu$  (Duggan *et al.*, 1957), the formation of adrenolutins and ethylenediamine conjugates makes possible quantitative differentiation of A and NA.

#### A. LUTINES

The yellow-green fluorescence developed when A solutions were treated with strong alkali was shown to be specific (Loew, 1918; Paget, 1930). Further studies indicated that oxygen was essential for the reaction and that NA had only 2% of the fluorescence of A (Gaddum and Schild, 1934). Attempts to utilize this reaction for quantitative purposes were unsuccessful, because of its instability. Formation of the fluorescent compound occurred more rapidly than the degradation; thus, fluorescence increases initially and then decreases after having reached a maximum. Identification of this fluorescent compound was achieved by a number of researchers (Ehrlén, 1948; Fischer, 1949; Lund, 1949a; Harley-Mason, 1950). The chemical constitution was established as 1-methyl-3,5,6-trihydroxyindole (THI).

Adrenaline (Fig. 2, I) is oxidized in alkaline solution to adrenochrome (Fig. 2, III), which has no fluorescence, through the formation of leucoadrenochrome (Fig. 2, II). The latter compound has as yet not been isolated, because of its instability. The fluorescent compound, adrenolutin

(Fig. 2, IV), was formed through rearrangement of adrenochrome (Fig. 2, III). The addition of ascorbic acid in the last step prevented further oxidation of the fluorescent compound, thus achieving a stable fluorescence (Ehrlén, 1948).

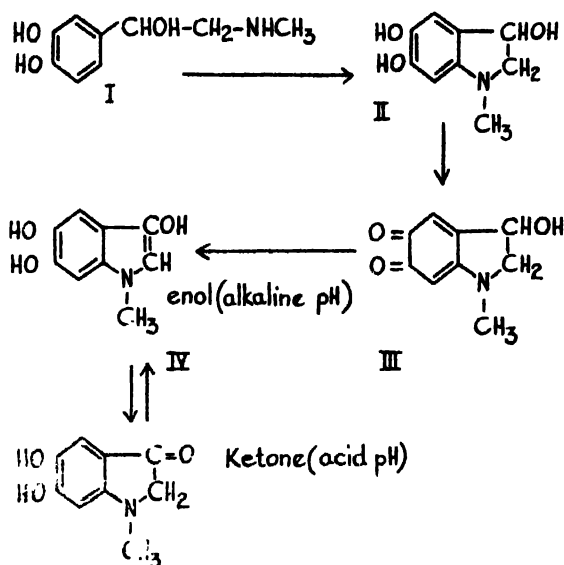


FIG. 2. Formation of a fluorescent compound by oxidation of adrenaline in strong alkali.

Acetylation with acetic anhydride-pyridine resulted in isolation of 3,5,6-triacetoxy-*N*-methylinole (Harley-Mason, 1950). The fluorescent compound was obtained by ether extraction after immediate neutralization of the alkaline solution (Fischer, 1949). Crystallization of the fluorescent compound was achieved in this fashion. The Belgian group also showed that adrenochrome may be isomerized to 3,5,6-trihydroxy-1-methylinole by treatment with zinc acetate in neutral solution (Fischer *et al.*, 1950). When strong alkali was added to oxygen-free adrenochrome, a change in color from yellow-green to yellow fluorescence occurred; the addition of acid changed the fluorescence to green (Lund, 1949b). These studies confirmed that the fluorescent compound was formed, by the addition of alkali to adrenochrome, by an isomeric transformation to 1-methyl-3,5,6-trihydroxyindole.

## B. ETHYLENEDIAMINE CONJUGATION

The demonstration of the fluorescent compound activated at 435 m $\mu$  by conjugation of adrenaline and ethylenediamine was shown to be

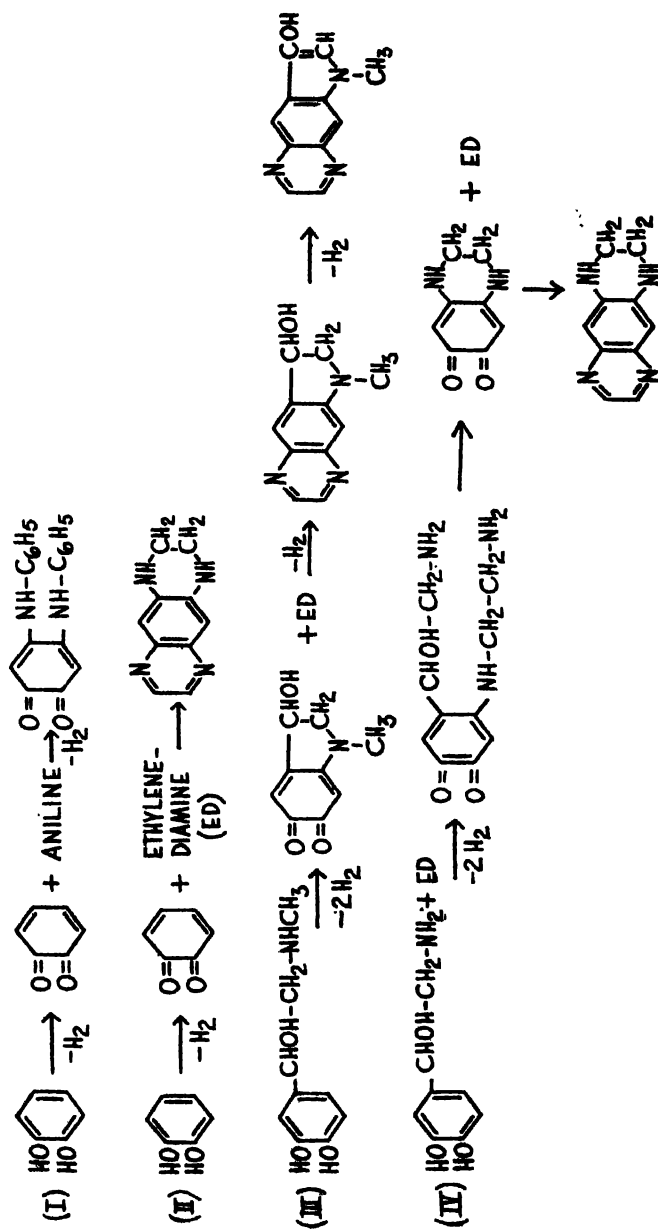


Fig. 3. Ethylenediamine conjugation with adrenaline and noradrenaline.

specific (Natelson *et al.*, 1949). Previous studies indicated that oxidized catechols form fluorescent condensation products with *o*-phenylenediamine (Pugh and Raper, 1927; Wallerstein *et al.*, 1947). The preferred reaction in an oxidative condensation was in the two positions *para* to the quinoid oxygens (Fig. 3, I). When substituents are present in the *para* position to the quinoid oxygens, a nonoxidative condensation takes place. In the case of ethylenediamine conjugation with catechol, both types of reaction take place (Fig. 3, II). With adrenaline rapidly cyclizing to adrenochrome, which has no *para* position to quinoid available for reaction, condensation takes place with quinoid oxygens (Fig. 3, III) (Hackman and Todd, 1953). The main product with NA is the same as that formed with catechol (Fig. 3, IV). This is explained by noting that, since NA cyclizes much more slowly than A quinone, there is conjugation of ethylenediamine in position 6. Then cyclization of the ethylenediamine side chain takes place with elimination of the  $\beta$ -ethanolamine portion of the NA molecules. This reaction occurs *only* if the hydroxyl group is on the  $\beta$ -carbon (Weil-Malherbe, 1959). Another mole of ethylenediamine then condenses with the quinoid oxygens. Various other reactions have been hypothesized as side products. Some investigators have reported several fluorescent derivatives in each of these reactions (Burn and Field, 1956; Nadeau and Joly, 1958; Young and Fischer, 1958). Weil-Malherbe contends that this may be due to the increase in the ratio of A and ethylenediamine used. He emphasizes that when the reaction is conducted under optimal conditions as described (Weil-Malherbe and Bone, 1952, 1954), the fluorescent spectra of A and NA are symmetrical curves with single peaks.

### III. Colorimetric Methods

Adrenaline is easily oxidized under various conditions to form adrenochrome. A number of tests using a dozen different oxidizing agents depend on this reaction (Munch, 1931), and, though these tests are not sensitive, they are fairly specific.

Other methods in which A acts as a reducing agent are more sensitive, but generally less specific. Other reducing substances must therefore be removed before the test can be applied. Naphthoquinone condensation with the primary amine allows measurement of NA in mixtures of A and NA.

## A. IODINE METHOD

Oxidation of preparations containing A and NA with iodine forms corresponding iodochromes, which have been utilized for the purposes of chemical assays (von Euler and Hamberg, 1949). The technique is based upon the differences in the rate of oxidation of A and NA at a pH of 4.0 with iodine. Adrenaline is completely transformed to its respective chrome in  $1\frac{1}{2}$  minutes, at a pH of 4.0, while only 10% of NA is oxidized at this same pH in the same time. At a pH of 6.0, both catechols are completely oxidized in 3 minutes. To stop the reaction at its prescribed time, an excess of 0.05 *N* thiosulfate is added. This reaction is carried out at pH 4.0 and 6.0, and the respective times of  $1\frac{1}{2}$  and 3 minutes. A blank is prepared without iodine and the colors developed are measured at 529  $m\mu$ . The colors developed at these pH values differ somewhat in that a blue tint is present at pH 6.0. Limitations of this method depend on the various concentrations of A and NA present in the mixture. Accuracy, in terms of standard deviation, is about 1% when single amines are measured. The standard deviation increases to 5–10% when one or the other of the amines is present in a concentration of 10–20% in the mixture. If either catechol is present in amounts less than 5%, the error is considerable. A column of neutral Permutit on which the mixture of catechol amines are adsorbed and then treated with iodine has been described (Lecompte and Fischer, 1949). A solution of iodine is passed through the column at a pH of 4.0 in 0.2 *M* potassium phthalate. Under these conditions, A is rapidly transformed into its iodochrome and washed out of the column without removing the NA. The procedure is repeated at pH 7.0, using 0.1 *N* iodine and 0.2 *M* phosphate buffer, and in 5 minutes NA is oxidized. The iodochrome is washed out with the phosphate buffer at a pH of 7.0. The color of the chromes are then measured separately in a colorimeter.

## B. PERMANGANATE METHOD

The use of permanganate as the oxidant avoids the inconvenience of obtaining color tints differences as observed with iodine (Suzuki and Ozaki, 1951). At a pH of 3.6, A is completely oxidized in 2 minutes with only 5–10% of the NA oxidized under the same conditions. At a pH of 5.6, both compounds are completely oxidized in 3 minutes. Then 0.1 ml. of permanganate reagent is added to aliquots of the same sample, and after 2 minutes at pH 3.6, and 3 minutes at pH 5.6, hydrogen peroxide is added to the mixture and subsequently diluted to



a 6-ml. volume. The amounts of A and NA are calculated by a given formula.

### C. ARSENMOLYBDIC ACID METHOD

Though this reaction is the most sensitive of the colorimetric methods, its main drawback is its lack of specificity (Whitehorn, 1935; Shaw, 1938; Raab, 1943). Considerable criticism has been made of this method because of erratic results obtained (Bloor and Bullen, 1941; Verly, 1948). Essentially, the method calls for the adsorption of A and NA on alumina which is then dissolved in sodium hydroxide. After 2 minutes, sulfite reagent is added. The mixture is then transferred to a tube containing arsenomolybdic acid reagent which has been previously heated in a water bath. The reaction is allowed to continue in a hot water bath for 5 minutes, cooled, and brought to volume with water. The blue color obtained is read 15–20 minutes later with a reagent blank and appropriate standards. The test is sensitive at 0.05  $\mu\text{g.}$  of A and 1.0  $\mu\text{g.}$  of NA.

### D. NAPHTHOQUINONE CONDENSATION

This method is based on the principle that the sulfonic acid group of 1,2-naphthoquinone-4-sulfonate can be replaced by a primary amine, usually yielding a colored compound (Auerbach and Angell, 1949). Secondary amines generally do not react under these test conditions. This makes it possible to determine the concentration of NA in the presence of A. The method consists primarily of the reaction of NA in 50- to 100- $\mu\text{g.}$  amounts with naphthoquinone reagent in a borate buffer with a pH of 9.6. This mixture is allowed to stand for 45 minutes at room temperature, with gentle shaking at regular intervals. Benzalkonium chloride is now added, followed by 10 ml. of toluene-ethylene dichloride. This mixture is again allowed to stand for 45 minutes, with gentle shaking at regular intervals. The solvent layer becomes a purplish-red, if NA is present, and should separate clearly. The colored layer is removed and read in a spectrophotometer at 540  $m\mu$ . A blank is run, containing about 1 mg. of A. The borate buffer diminishes the side reactions of catechol groups. Benzalkonium chloride yields a fatty salt which is essentially an acetic dye. The colored compound has a broad band maximum of 530–560  $m\mu$ . Extraction with toluene-ethylene dichloride, eliminates interference by such compounds as dihydroxyphenylalanine and dihydroxyphenylethylamine.

#### IV. Fluorometric Methods

The introduction of fluorometers and the understanding of the chemistry of fluorescent compounds produced by catechol amines have resulted in the publication of a number of methods for analyses of blood, urine, and tissues. The principal methods now in use are based upon the formation of lutins (Lund, 1949a, b) and ethylenediamine conjugates (Weil-Malherbe and Bone, 1952, 1954).

##### A. TRIHYDROXYINDOLE (THI) METHOD

Urine (von Euler and Lishajko, 1959), blood (Cohen *et al.*, 1959), and tissues (Shore and Olin, 1958) may be estimated by measuring the fluorescence produced by the formation of lutins (see Section II, A). The essential feature of all these methods requires the adsorption of catechol amines on alumina, either by a "batch" or a column method, with subsequent elution by such acids as sulfuric, acetic, and oxalic. Determinations of mixtures of A and NA are achieved by differential oxidation at pH 3.0 and 6.0, or by the use of multifilters and the spectrophotofluorometer. Oxidation is achieved by using such agents as manganese dioxide, iodine, and potassium ferricyanide. Stabilization of the fluorescent compound with ascorbic acid (Ehrlén, 1948) then makes possible the estimation of quantitative aspects of the fluorescence with a suitable instrument.

Various modifications of the method are thus possible and the variability in results is due to a lack of standardization of the method. There is, however, some agreement that the alumina column method is preferable to the "batch" method; that acetic acid and oxalic acid are more selective of the amines and do not include such interfering compounds as catechol acids, as in the case with sulfuric acid (Weil-Malherbe, 1959); that iodine does not quench the fluorescence as much as ferricyanide (Crout, 1959). Instrumentation has not been standardized and arguments have been given in support of the use of a band of exciting light that is sufficiently shielded, but which covers most of the solution, rather than a single beam of light concentration in a small area of the solution. The emitting light should be measured from the bottom of the tube, so that only one area of resistance of glass is made with the open end free (Weil-Malherbe, 1959). The requirements of a good spectrophotofluorometric assay includes a minimum of scatter and quenching. Light scatter may be reduced by seeing that no solid particles, either from the reagents or from a fine precipitate of aluminum salts, are

present. Quenching may be tested by showing that the addition of a standard solution to the extract to be assayed results in an additive effect without any loss of fluorescence. For example, the titer of the extract to be measured, plus the standard separately, should be equal to the same degree of fluorescence as when the extract and the standard are mixed. It appears that the THI method involves more light scatter and quenching than the EDA method. The blank also offers a problem in the THI method. There is a tendency for the blank to rise with time, at a faster rate than the samples themselves (Crout, 1959). A discrepancy of 5 units may appear in 30 seconds, due to the decomposition of ascorbic acid which is used in strong alkali for stabilization of the fluorescence.

The THI method may be performed with 25 ml. of urine (von Euler and Lishajko, 1959). The method consists essentially in boiling and filtering the urine to which is added the disodium salt of ethylenediamine; the mixture is adjusted to pH 8.3 with sodium hydroxide. A column of 10-mm. bore is prepared with 1.0 gm. of aluminum oxide. The mixture is then carefully added to the column and allowed to pass through at a rate of 1 to 2 ml. per minute. The column adsorbs about 95% of the catechol amines. Elution is achieved with the addition of 0.25 *N* acetic acid to the column. The eluate is oxidized with potassium ferricyanide after adjusting to pH 6.2-6.3 with ammonia. Lutins are formed with treatment with strong alkali-containing ascorbic acid. Filters are used (Cohen and Goldenberg, 1957) for analysis of A and NA with a Coleman fluorometer. Filter set A consists of a primary interference filter 395  $m\mu$ , for excitation, combined with an Ilford Bright 623 secondary filter (peak transmission 490  $m\mu$ ). This filter combination measures A and NA with equal fluorescence intensity. Filter set B, composed of a 436- $m\mu$  interference filter for excitation and a Corning 3486 secondary filter (peak transmission 354  $m\mu$ ), gives 3 times as high a fluorescence for A as NA, with a suitable blank; and, formula calculations may be made for A and NA (Cohen and Goldenberg, 1957).

Essentially, the same method may be used for plasma analysis (Cohen and Goldenberg, 1957; Price and Price, 1957).

#### B. ETHYLENEDIAMINE CONJUGATION

Ethylenediamine conjugation reaction (EDA) has been utilized for blood (Weil-Malherbe and Bone, 1952, 1954; Mangan and Mason, 1958; Manger *et al.*, 1959), urine (Weil-Malherbe and Bone, 1957), and tissues (Blaschko *et al.*, 1955; Montagu, 1956). Great variability in A data on blood has appeared, using this method (Table I). The

urine method is rather complicated and requires the additional use of the THI method because of the presence of dopamine in the urine. Various modifications of the EDA method have been extensively used (Weil-Malherbe and Bone, 1954; Aronow and Howard, 1955; Richardson *et al.*, 1956; Zileli *et al.*, 1957; Mangan and Mason, 1958; Millar 1956; Manger *et al.*, 1959).

The EDA method gives higher values than the THI method (Table I). This may be explained in part by the fact that antioxidants are

TABLE I  
FLUOROMETRIC METHODS

No. of subjects	Adrenaline ( $\mu\text{g./liter}$ )	Noradrenaline ( $\mu\text{g./liter}$ )	Reference
Trihydroxyindole (THI) method			
65	$0.06 \pm 0.05$	$0.30 \pm 0.07$	Cohen and Goldenberg (1957)
12	$0.01 \pm 0.07$	$0.34 \pm 0.15$	Price and Price (1957)
4	$0.00 \pm 0.01$	$0.20 \pm 0.19$	de Valk and Price (1956)
75 ♂	$0.07 \pm 0.01$	$0.35 \pm 0.01$	Vendsalu (1960)
50 ♀	$0.06 \pm 0.01$	$0.35 \pm 0.01$	Vendsalu (1960)
Ethylenediamine (EDA) conjugation			
22 ♂	$1.18 \pm 0.21$	$5.29 \pm 1.10$	Weil-Malherbe and Bone (1953)
21 ♀	$1.46 \pm 0.38$	$5.16 \pm 0.74$	Weil-Malherbe and Bone (1953)
7	$0.14 \pm 0.21$	$3.96 \pm 1.7$	Manger <i>et al.</i> (1954)
6	$0.4 \pm 0.2$	$2.1 \pm 1.3$	Aronow and Howard (1955)
17	$0.097 \pm 0.14$	$2.74 \pm 1.4$	de Valk and Price (1956)
12	—	2.09 <sup>a</sup>	Mangan and Mason (1958)
6 ♂	$0.18 \pm 0.16$	$4.4 \pm 1.1$	Manger <i>et al.</i> (1959)
6 ♀	$0.51 \pm 0.22$	$2.4 \pm 0.54$	Manger <i>et al.</i> (1959)

<sup>a</sup> Mean value.

not added to the blood with the THI method. The large concentration of ascorbic acid may be responsible for quenching (Manger 1959). An important modification of the EDA method requires the use of thio-sulfate which inhibits the fluorescence of NA, but not A, thus avoiding the measurement of mixtures of catechol amines (Manger, 1959). This modification is less sensitive only because 2 aliquots are used for each determination. It must be pointed out that the EDA and THI methods have been reported to give essentially the same values (Weil-Malherbe and Bone, 1958), but these methods still do not agree with bioassay methods.

The EDA method for plasma determinations require 15 ml. of

blood (Mangan and Mason, 1958). The sample is drawn into a syringe containing 5 ml. of fluoride-thiosulfate solution. The plasma is then separated by centrifugation. The presence of fluorescent material presumed to be A and NA in red cells requires considerable care at this point (Weil-Malherbe and Bone, 1954). The plasma is added to equal parts of sodium acetate buffer and the mixture is adjusted to pH 8.4 with sodium carbonate. The column stem used is 5 mm. in bore, requiring about 0.5 gm. of aluminum oxide. Mild suction is required to allow an adequate flow of fluids through the column. The plasma-acetate mixture is then added to the column, followed by 5 ml. of acetate buffer and 5 ml. of glass-distilled water. Some difficulty may be encountered due to blocking of the column by plasma lipids. This may be overcome by displacing the precipitate with a glass rod. The adsorbed A and NA may then be eluted by passing 5 ml. of 0.2 *N* acetic acid through the column, followed by 5 ml. of water. Ethylenediamine dihydrochloride solution is then added to the eluate, followed immediately with ethylenediamine and the reaction carried out at 50°C. for 20 minutes. At the end of this period, the samples are placed in a cold water bath and, after cooling for 5 minutes, the mixture is saturated with sodium chloride and extracted with isobutanol in a mechanical shaker. The isobutanol phase is transferred to a fluorometric cuvette and fluorescence determined by use of a formula (Mangan and Mason, 1958).

The marked discrepancy reported in the values for concentration of A and NA in systemic blood by the EDA and THI methods have been of major concern. Based on physiological considerations, it is quite clear that the values given by EDA procedures are high. The recent extensive studies (Cohen *et al.*, 1959; Vendsalu, 1960) have further reaffirmed the validity of the THI method. Vendsalu (1960) uses quite a different extraction procedure (Dowex 50-X4) with the Aminco spectrofluorometer. More recently, evidence has been presented by Weil-Malherbe indicating the presence of catechol acids with use of the EDA procedure, which could account for the high values obtained (von Euler, 1961). Resolution of this problem should soon be achieved.

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## Chapter 9

# Chemical Assay of Thyroxine-like Materials<sup>1</sup>

S. B. BARKER

I. Introduction . . . . .	351
A. Historical . . . . .	351
B. General Consideration of Procedures . . . . .	353
II. Separation of Iodo-Compounds . . . . .	354
A. Free, or Noncovalent-Bound Compounds . . . . .	355
B. Peptide-Linked Iodoamino Acids . . . . .	367
III. Chemical Methods of Determination . . . . .	369
A. Iodine . . . . .	370
B. Amino Acid Functions . . . . .	375
C. Phenolic Functions . . . . .	376
IV. Physical Methods of Determination . . . . .	378
A. Ultraviolet Spectrophotometry . . . . .	378
B. Polarography . . . . .	379
C. Radioactivity . . . . .	380
V. Summary . . . . .	382
References . . . . .	382

## I. Introduction

### A. HISTORICAL

The determination of substances associated with thyroid activity started with Emil Baumann who, in 1896, first demonstrated the presence of an insoluble form of iodine in the thyroid gland. Interest in this area had previously been aroused by the earlier work of Fyfe (1819), that burnt sponge, used as a folk remedy for goiter, contained iodine, of Coindet (1820), that iodide itself could be used to treat goiter, and of Chatin (1850) associating endemic iodine deficiency with goiter. As one reads the extensive papers of Baumann (1896), Oswald (1899), Hutchison (1896), and others of that day, he senses frustrations of methodology similar to those facing thyroid workers even now. At that time, there was no information about specific iodoprotein compounds and Baumann could only show close association of the iodine with protein and a clear differ-

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entiation from inorganic iodide. It was not long after this that Hutchison (1896) could show a correlation between the therapeutic activity of thyroid hydrolytic products and their iodine content.

With the establishment of the value of thyroid replacement therapy in myxedema, also late in the nineteenth century (Murray, 1891), determination of thyroid materials became of practical importance. Revision VIII of the U.S.P. (Pharmacopeia, 1905) first gave a test for combined iodine in desiccated thyroid substance, to be performed by  $\text{KNO}_3$  oxidation in fused alkali and extraction of free  $\text{I}_2$  into chloroform yielding a "decided pink to violet coloration." By revision IX (Pharmacopeia, 1916), the assay had been made quantitative, involving a final titration of liberated  $\text{I}_2$  with  $0.02\text{ }N\text{ Na}_2\text{S}_2\text{O}_3$ . The present limits of 0.17–0.23% iodine in combined form and freedom from inorganic iodide were prescribed at that time. The difficulties of correlating such thyroid iodine determinations with physiological activity will be discussed later.

It is noteworthy that, as early as 1905, Wheeler and Jamieson had shown that Drechsel's Jodgorgosäure, isolated from the horny skeleton of coral following alkali treatment, was diiodotyrosine. In spite of much interest in iodotyrosine (cf. Wheeler and Mendel, 1909), when Kendall obtained the first crystalline thyro-active compound in 1919, he considered it a triiodooxyindole derivative, giving it the name "thyroxine." This situation arose partly from misinterpretation of a color test and partly from erroneous nitrogen analyses, leading to the belief that each molecule contained only three iodine atoms. Not until Harington and Barger (1927) gave the complete proof of thyroxine structure and also isolated 3,5-diiodotyrosine from the thyroid gland was the relationship between thyroxine and diiodotyrosine made clear.

This theory of production of diiodotyrosine, followed by condensation of two diiodotyrosines to thyroxine, elaborated by Johnson and Tewkesbury (1942), has been experimentally confirmed by observations on incorporation of radioiodine into thyroid protein *in vivo* (Mann *et al.*, 1942) and *in vitro* (Morton and Chaikoff, 1943) as well as nonenzymatic *in vitro* thyroxine formation in proteins containing tyrosine (Roche *et al.*, 1947). A possible specific importance of "protective" combination of the carboxyl and amino groups of tyrosine becomes apparent upon comparison of the increased yields of thyroxine derivatives obtained from coupling of free diiodotyrosine (4%), N-acetyldiiodotyrosine (25%), and N-acetyldiiodotyrosylglutamic acid (35%) (Pitt-Rivers, 1948). Still more recently, Pitt-Rivers and James (1958) have obtained as much as 50% coupling using the more complex peptide  $\alpha$ -(N-acetyl)- $\epsilon$ -(N-acetyldiiodotyrosyl)-lysine.

A detailed historical background of thyroxine analyses was presented



by Pitt-Rivers (1950). In the 10 years since her review, much new material has been brought to light, especially that involving an increased attention to iodinated substances other than thyroxine. This development was largely catalyzed by the discovery of the biologically more active 3,5,3'-triiodothyronine (3,5,3'-T<sub>3</sub>) (Gross and Pitt-Rivers, 1952). This compound had been postulated earlier by Hird and Trikojus (1948) on the basis of chromatographic studies of iodoprotein hydrolyzates, but bioassay of isolated material showed low activity.

In addition to 3,5,3'-T<sub>3</sub>, two other expected iodothyronines, 3,3',5'-triiodothyronine and 3,3'-diiodothyronine, have been reported present in rat thyroids and blood (Roche *et al.*, 1955, 1956), undoubtedly representing the combination of 3,5-diiodotyrosine (DITy) plus 3-monoiodotyrosine (MITy) and MITy plus MITy, respectively. The absence of 3,5-diiodothyronine and thyronine points to the importance of iodination on both tyrosine molecules as a preliminary to coupling. Following administration of 3,5,3'-T<sub>3</sub> to dehepatized dogs, Flock *et al.* (1960) have encountered the sulfate ester of 3,3'-T<sub>2</sub> in plasma and urine as the chief metabolite. Other than this report, neither 3,3'-T<sub>2</sub> nor 3,3',5'-T<sub>3</sub> has been found in large quantities, either preformed or as metabolic products of other compounds. Neither can be considered as metabolically active "thyroid hormones," but the isomeric T<sub>3</sub> has been found to have an interesting thyroxine-blocking effect (Barker *et al.*, 1960).

#### B. GENERAL CONSIDERATION OF PROCEDURES

From what has been presented, it can be appreciated that the present status of the thyroid hormone analytical situation is one of deciding between specificity and sensitivity of determination. A combination of the two requires considerable extra care and labor, if, indeed, it can be accomplished at all.

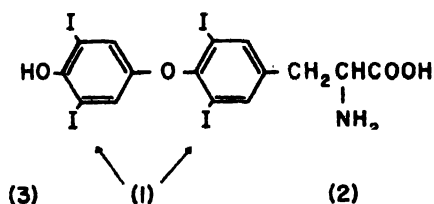


FIG. 1. The structure of thyroxine (T<sub>4</sub>) (see text).

Examination of the structure of T<sub>4</sub> (Fig. 1) and similar compounds reveals three chemical characteristics which have been used for analyses: (1) the organic iodine, (2) the  $\alpha$ -amino acid side chain, (3) the 4'- or

4-phenolic hydroxyl. The first is the only unique function and can be made the most sensitive, either by chemical or radioactive tracer determinations. The presence of the alanine side chain enables the use of the conventional ninhydrin reaction, but the large iodinated diphenyl ether substituent interferes to such an extent that much more material is required than with most other  $\alpha$ -amino acids. Several reactions involve the phenolic hydroxyl group, especially as modified by the adjacent iodines.

Since none of these reactions is truly capable of differentiating one thyroxine-like substance from another, a great deal of attention has been paid in the literature to methods for the physical separation of the 40 or so compounds of interest, including the analogs with fatty acid side chains. For the purpose of convenience, the ensuing discussion will first consider methods of separation and then the actual determination of thyroxine compounds. In this way, comparisons can be made of different workers' approaches to the various major problems. The large number of reports on technique imply that none is as yet completely satisfactory, although continued experiences with any fundamentally sound procedure reveals the minutiae of careful handling required for consistent results more than any light-hearted skipping from one to another. The present account can do nothing more effective than call attention to various types of methods, including only a small amount of detail. No attempt will be made to discuss or even to cite every procedure reported.

## II. Separation of Iodo-Compounds

The iodoamino acids are found in two forms: "free" or in peptide linkages. Every tissue must have some free material, representing its equilibrium with the plasma. However, in the presence of soluble protein, such as albumin and certain globulins, the iodoamino acids are more or less completely bound to the protein. This is not a covalent bond, since it can be split by simple extraction with various organic solvents, in contradistinction to the strong methods required to disrupt the peptide link, but it is strong enough to persist during electrophoresis.

It is obvious that careful consideration should be given to the purpose of any series of analyses in order to carry out the proper preliminary steps. Often these are fully as important as the final delicate determination. Table I outlines the steps in handling the two types of iodoamino acid combinations. The ensuing discussion will follow Table I in each instance down to the common item of actual determination. For convenience, there will be some cross-over where this seems most logical.

TABLE I  
HANDLING OF VARIOUS TYPES OF IODOAMINO ACID COMBINATIONS

Nature of linkage	Examples	Procedures
Free or loosely bound	Plasma PBI Tissue PBI	Electrophoresis PBI procedure Solvent extraction Chromatography Determination
Peptide-linked	Thyroglobulin Other natural iodoproteins, normal and abnormal Synthetic iodoproteins	Isolation of protein Hydrolysis Separation of amino acids Determination

#### A. FREE, OR NONCOVALENT-BOUND COMPOUNDS

##### 1. *Electrophoresis*

This type of preliminary separation has been used especially in investigations of plasma levels of  $T_4$  and  $T_3$  and has been very important in developing the concept of transport by loose binding with specific plasma proteins (Gordon *et al.*, 1952). The conventional Tiselius barbiturate buffers at pH 8.6 with either paper or starch support showed a  $T_4$ -inter- $\alpha$ -globulin complex, and a  $T_4$ -albumin complex forming with larger quantities of  $T_4$  (above 15  $\mu\text{g.}\%$ ) (Robbins *et al.*, 1954). Since  $T_3$  was easily displaced from any combination with the  $\alpha$ -globulin, it was considered a specific "thyroxine-binding protein" (TBP), or "thyroxine-binding globulin" (TBG).

More recent electrophoretic studies (Ingbar, 1960), employing tris hydroxymethyl aminomethane or other nonbarbiturate buffers, have revealed the presence of a "prealbumin" with a considerably greater  $T_4$ -binding ability, but with less  $T_4$  specificity than claimed for the  $\alpha_2$ -globulin. Considerable amounts of Tetrac and Tetraprop added to serum can be bound to the prealbumin, but not to TBG; 3,5,3'-triiodothyronine is not bound to the prealbumin, but Triac and Triprop are. Most of these analyses have been done with added  $I^{131}$ -labeled  $T_4$  or  $T_3$ , or with plasma of animals or patients after  $I^{131}$  administration.

As pointed out by Ingbar (1960), the emergence of the additional thyroxine-binding prealbumin is so recent that its implications on physiological thyroxine transport cannot yet be fully evaluated. However, it should be kept in mind that  $T_4$ , even at 1000  $\mu\text{g.}\%$ , is entirely bound to protein, in contrast to  $T_3$ , and that albumin, with only a low molecular binding ability, may be quantitatively important because of its higher concentration (Robbins *et al.*, 1954).

By means of the extremely new technique of immunoelectrophoresis, Clausen and Munkner (1960) report finding added radioactive  $T_4$  and  $T_3$  associated with the three known lipoproteins of human serum, in the pre-albumin, albumin, and  $\alpha$ -globulin areas. No specific binding could be detected between  $T_4$  or  $T_3$  and any of the glycoproteins of human serum, such as the thyroxine-binding  $\alpha$ -globulin has been thought to be.

Such results indicate such a lack of certainty about  $T_4$  binding as to cast considerable doubt on the otherwise ingenious estimation of plasma  $T_4$  by means of the redistribution of added radioactive  $T_4$  between albumin and the inter- $\alpha$ -globulin TBP area (Ekins, 1960).

Thyroxine-binding activity has been demonstrated in a variety of body fluids, such as cerebrospinal fluid (Alpers and Rall, 1955) lymph, and joint and ascites fluid (Freinkel *et al.*, 1957). Not as much investigation has been given to solid tissues, undoubtedly because of difficulties in preparation of consistent protein solutions. Tata (1958) has demonstrated the existence of skeletal muscle TBP with quite different electrophoretic characteristics than the serum TBP from the same species. It may be inferred from the finding (Etling and Barker, 1959) of rapid uptake of thyroxine by rat kidney that binding also occurs in this tissue. A more specific study by Freinkel *et al.* (1957) led to the conclusion that the distribution of  $T_4$  between protein-containing solutions and slices of liver, heart, and kidney could be formulated in terms of reversible binding on the part of compounds of both tissue and extracellular fluid.

It is quite possible that combinations of saline extraction, ultracentrifugation, and electrophoresis applied to other tissues might yield important information concerning association of  $T_4$  with specific proteins.

A procedure for evaluation of  $T_3$  uptake by peripheral cells has been proposed by Hamolsky *et al.* (1957, 1959). This involves a 2-hour incubation at 37°C. of an aliquot of whole blood with radioiodine-containing  $T_3$ . Following this, the blood is centrifuged and the red blood cells (R.B.C.) washed 5 times with a tenfold volume of saline. Activity remaining in the packed cells is determined in a well counter and the results expressed as per cent uptake by the R.B.C., on the basis of a hematocrit reading of 100. Excellent separation is reported between low uptake in known hypothyroid patients (9.5%) and euthyroids (14.6%), and slight overlap between euthyroid and hyperthyroids (23.1%). Although the theoretical basis for this test is not secure, it is presumably the result of differing availability of  $T_3$ -binding sites. An attempt by Friis (1960) to correlate with  $T_4$  binding was not successful.

Besides having been shown to be statistically valid, the procedure has much to recommend it as being manipulatively simple, avoiding exposure of the patient to radioactivity, and at the same time being, at

least partially, a functional evaluation of peripheral cellular activity. Mead (1960) has called attention to discrepancies produced by the prolonged washing after incubation, since no stable equilibrium is reached. On theoretical grounds, even assuming that as much as 10% of the packed R.B.C. volume was plasma after the first centrifugation, one washing with 10 volumes of saline per volume of R.B.C. should leave only about 0.05% of the original activity contaminating the R.B.C., and two such washings about 0.0005%. These low errors of contamination make re-examination of the important details of washing imperative before the technique becomes completely established.

Electrophoresis in 0.05 *M* ammonium carbonate solution at a fairly high direct current of 500 volts can be used to separate iodide and the fatty acid analogs of  $T_3$  and  $T_4$  from  $T_3$  and  $T_4$ . Roche and Michel (1960) have also moved the sulfate ester of  $T_4$  away from  $T_4$ -glucuronide by this procedure. The greater mobility of the substances with a higher net charge enables such a separation by their migration toward the anode.

## 2. Plasma or Serum "Protein-Bound Iodine" Procedure

*a. Protein Precipitation and Washing.* This is an important preliminary to the clinical determination of the "protein-bound iodine" (PBI) of the blood plasma as well as to certain other determinations of protein-linked  $T_4$ ,  $T_3$ , etc. It is necessary not to confuse the physiological, loosely associated TBP (globulin or prealbumin) of plasma with the coprecipitation of  $T_4$  and  $T_3$  (but not MITy or DITy) with denatured protein or with the peptide-linked  $T_4$ ,  $T_3$ , MITy and DITy found in thyroglobulin preparations, no matter how superficially similar such preparations may seem. The clinical applicability of determining the level of iodine precipitated with the serum proteins is based on the fortunate fact that  $T_4$  and  $T_3$ , the physiologically active materials found in the plasma, are so precipitated, and that inorganic iodide is not.

Another confusion in terminology should be avoided, namely that all PBI is hormonal. PBI is a purely descriptive term related to the determination of iodine associated with protein. In the thyroid gland, except for traces of free iodoamino acids, all the organic iodine is contained in peptide-linked compounds, and hence is precipitated with protein, even though only about 30% is  $T_4$  plus  $T_3$ . It can easily be shown that a host of organic compounds containing C—I covalent bonds, such as iodopyracet (Diodrast®), tetraiodophenolphthalein (Iodeikon®), acetrizoate (Urokon®), diatrizoate (Hypaque®), iophenoxic acid (Teridax®), used widely in roentgenographic contrast media, are coprecipitated with any proteins present (Astwood *et al.*, 1957; Cassidy, 1960; Heijdemann and Lindeboom, 1958). Some evidence suggests that the blood levels follow-

ing their administration by practically any route are truly associated with plasma proteins in the circulation. They are not metabolic stimulants [Barker *et al.* (1951a and unpublished) have actually found some to block the peripheral activity of  $T_4$ ], regardless of being protein bound.

The most effective methods of protein precipitation are the zinc hydroxide (Somogyi) and the trichloroacetic acid (TCA). For the former, 1.0 ml. of plasma or serum in a centrifuge tube is diluted with 7 ml. of distilled water and 1.0 ml. of 10% zinc sulfate added. The contents of the tube are mixed with a fine glass stirring rod or with a Vortex® stirring device. One millimeter of 0.5 N NaOH previously standardized against the zinc solution (Barker *et al.*, 1951b) is added and the solutions thoroughly mixed to ensure even distribution of the alkali and complete precipitation of the proteins.

The TCA precipitation is simpler, involving addition of 10 ml. of 5% TCA to 1 ml. of plasma or serum, followed by thorough mixing. With either reagent, the precipitated protein is centrifuged down and the supernatant solution poured off. The zinc complex can be washed 3 times by resuspending the precipitate in 10 ml. distilled water and centrifuging, which will remove better than 99% of any inorganic iodide present. The TCA precipitate can be similarly washed with 5% TCA, but not with water, which redissolves the protein. However, Astwood (personal communication) has found that there is a loss of PBI and recommends omitting this step.

Either of these two methods of precipitating proteins can easily be applied to other biological fluids or tissues. For the latter, preliminary thorough grinding in water with the Potter-Elvehjem glass homogenizer (Potter, 1957) is recommended prior to adding reagents.

*b. Incineration.* Since the usual amounts of organic iodine present in biological material are about 5  $\mu\text{g.}/100\text{ gm.}$  (wet weight) there is about a four million-fold excess of organic material present which can interfere with the quantitative determination of iodine. Even in the thyroid gland itself, where the concentration of iodine may be a thousand times higher, it is usually necessary to destroy the excess organic material. This, of course, eliminates any possibility of differentiating various iodine-containing compounds, so all necessary separations must have been done previously.

Destruction of organic linkages can be done by alkaline ("dry-ash") or strongly acidic ("wet") reagents. The former are the older but still offer some advantages, especially in the ease of control. In Kendall's 1914 procedure, incineration with NaOH was carried out in an open crucible over a flame. A modern procedure (Barker *et al.*, 1951b) is to oven-dry a slurry of the tissue macerated in 10%  $\text{Na}_2\text{CO}_3$  or NaOH contained in a

test tube, and then to ash it in a thermostatically controlled muffle furnace, requiring no direct attention, except for readmission of air to the furnace (Foss *et al.*, 1960).

If sulfuric-dichromate (Barker, 1948) or sulfuric-permanganate (Riggs and Man, 1940) acid digestions are used, it is best to remove the iodine by distillation. This requires a special apparatus plus considerable technical skill and constitutes the most difficult stage of this type of determination. Because of this complication, there has been much searching for a mixture sufficiently powerful to eliminate organic material, but nonreactive to enable direct determination of formed iodide without distillation. A combination of chloric and chromic acids seems to fulfill these requirements.<sup>2</sup> The precipitated protein from 0.5 ml. of plasma or serum is digested in heavy-walled, 40-ml. Pyrex centrifuge tubes with 0.2 ml. of 0.5% sodium chromate plus 6 ml. of 28% chloric acid, prepared as described by Zak *et al.* Digestion can be carried out without attention for the first 1½ hours in a 175° sand bath or metal heating block. After that time, 2–3 drops of chloric acid should be added to each tube every 5 or 10 minutes for another hour to maintain the chromate catalyst in the oxidized state. The green color of reduced chromium should not appear.

*c. Removal of Iodide.* Foss *et al.* (1960) have recently shown that extraction of iodide from the alkaline ash can be performed with water, thus eliminating loss of iodine during foaming when acid is added directly to the dry carbonates. Insoluble material can be packed by centrifugation and the iodide-containing supernate poured off for analysis.

If the chloric acid wet digestion is used, simple dilution to an appropriate volume is adequate, but with sulfuric-chromic acid combinations, distillation into a reducing solution is necessary. Since arsenite is to be used later in the colorimetric analysis for iodide, this is preferable to the bisulfite earlier employed in the trap of the all-glass still (Barker and Lipner, 1948). Spitzzy *et al.* (1958) have described a flask for digestion which later is attached through a ground-glass joint to a diffusion apparatus.

### 3. Direct Solvent Extraction

As a forerunner of some of the more refined extraction methods used today, Leland and Foster in 1932 showed that T<sub>4</sub> could be separated with *n*-butanol from DITY even in the highly alkaline solution from hydrolysis of thyroid preparations. A more complete extraction of T<sub>4</sub> into butanol was achieved by lowering the hydrolyzate pH to 3.5 (Blau, 1933).

<sup>2</sup>The author is indebted to Dr. E. B. Astwood for details of his modification of the procedure of Zak *et al.* (1952), only partially reported in his Ciba paper (Astwood *et al.*, 1957).

Although much of the DITY was also extracted in acid, washing the butanol with 4 *N* NaOH–5% Na<sub>2</sub>CO<sub>3</sub> removed this. This type of butanol extraction was employed by many workers and was proposed by Man *et al.* (1951) as the basis of a more specific serum “hormonal iodine” determination than the total PBI. One major hope, that butanol extraction might separate true hormonal PBI from the nonphysiological organic iodine substances used roentgenologically, has not been realized, because of a lack of adequate differential solubility.

For this reason, plus the somewhat more complicated manipulations required, butanol-extractable iodine determinations have never reached the clinical popularity of the PBI. However, the relative ease of concentrating extracts representing a considerable volume of serum has appealed to those examining serum-iodinated material in qualitative detail. For instance, Block *et al.* (1958) have extracted 20 ml. of acidified serum containing “a little” thiouracil with 7–10 volumes of butanol for 2–3 minutes at 100°. The suspension was at once cooled in ice and filtered. The precipitated protein was washed with butanol. The hot butanol extraction has also been applied to the Somogyi zinc hydroxide-precipitated protein.

The butanol extracts were made alkaline with 7.5 *N* NH<sub>4</sub>OH and concentrated *in vacuo* to about 10 ml. at a temperature not specified, but presumably less than 40°C. The salts and other material insoluble in the resulting dry butanol were filtered and washed with water-free butanol. The combined butanol solutions were reduced *in vacuo* to less than 1 ml., made up to 5 ml. with fresh butanol, and 5–15 ml. of CHCl<sub>3</sub> were added. Three successive extractions were then carried out with 50, 40, and 40 ml. of 1 *N* NH<sub>4</sub>OH, emulsions being broken by centrifugation. The combined aqueous layers were taken to dryness *in vacuo* and redissolved in methanol–1% NH<sub>4</sub>OH. This solution was then used for paper chromatography.

The authors reported 92% recovery in the final extract of radioactivity present in the serum of a patient 72 hours after administration of 125  $\mu$ c. of NaI<sup>131</sup>. Addition of analyzed mixtures of radioactive T<sub>4</sub>, T<sub>3</sub>, and I<sup>–</sup> gave recoveries of 80–97% in these fractions with no evidence for production of MITY or DITY. For further recovery tests, analyzed mixtures rich in radioactive iodotyrosines were added to sera and processed. Kono *et al.* (1960) report the use of a similar acid-butanol extraction of serum without the use of heat and avoiding addition of thiouracil. Specific comment on the desirability of avoiding thiouracil will be made later, under paper chromatography.

Tata (1960) has reported alteration of T<sub>4</sub> to an inorganic iodide-like material when T<sub>4</sub> in a nonaqueous solvent is diluted with a large volume



of water during exposure to light. This was interpreted as a combination of ionization of the phenolic hydroxyl and splitting of iodide. The observation of return of this unknown to  $T_4$  with time, despite a large dilution factor, rules out reassociation of inorganic iodide. Galton and Ingbar (personal communication), although able to confirm partial reversibility, have found extensive deiodination of  $T_4$  to take place in dilute solutions without regard to light exposure, unless protein is added. Whenever chromatography of solutions alone yields unexpected amounts of iodide, this effect must be suspected.

#### 4. Column Chromatography

Column and paper chromatography both are continuous multiple-extraction techniques operating with much higher efficiency than the gross extraction just discussed. They both achieve a far greater degree of quantitative separation of closely related compounds, such as the iodothyronines from the iodotyrosines, but involve considerably more complicated techniques and hence are most often used when detailed separations are essential. In most instances, a cleaner separation of larger quantities of substances of interest can be obtained by column chromatography than by paper. It is, however, slower and more cumbersome, usually requiring a fraction collector. It will doubtless continue to be used less frequently than paper chromatography.

Gross and Pitt-Rivers (1953) effected the separation of  $T_3$  from  $T_4$  on a kieselguhr column with 0.5 *N* NaOH as the stationary phase and 20% chloroform in *n*-butanol as the moving phase. Thyroxine was eluted considerably ahead of  $T_3$ , enabling clean separation.

The use of a kieselguhr column for separation of iodinated compounds was extended by Braasch *et al.* (1954) to MITy and DITy in extracts of thyroid gland hydrolyzates. In following the general procedure of Gross and Pitt-Rivers, the first part of the column consisted of 6 gm. of Johns-Manville "Super-cel hyflo" mixed with 4.8 ml. of 0.5 *N* NaOH previously equilibrated with 25%  $CHCl_3$  in *n*-butanol. The same  $CHCl_3$ -butanol was then added until a thin slurry was achieved. This was degassed and packed in a 13-mm. glass tube to a height of 15 cm., using a perforated glass disk. On top of this was packed 0.3 gm. of Gooch crucible asbestos to which was added an alkaline solution of the mixture to be separated. The upper half of the column was another 5 gm. of kieselguhr mixed with 4 ml. 0.5 *N* NaOH saturated with 20%  $CHCl_3$  in *n*-butanol.

When the 20%  $CHCl_3$ -butanol saturated with 0.5 *N* NaOH was passed down the column at a constant rate of 1 ml./5 minutes,  $T_4$ ,  $T_3$ , and  $I^-$  were removed separately, in that order. The solvent mixture was

then changed to *n*-butanol:*n*-propanol (9:1) again saturated with 0.5 *N* NaOH. This eluted  $T_4$ - and  $T_3$ -conjugates, when present, then DITy, followed by MITy.

Kennedy and Purves (1956) introduced a stepwise gradient elution of these same five iodine compounds from a kieselguhr column. A 70:30 *tert*-butanol:cyclohexane mixture removed  $T_4$  in the first twenty 3-ml. fractions. Changing to 80:20 yielded  $T_3$ , to 90:10 the  $I^-$ . Replacing the *tert*-butanol with *n*-propanol to make 90:10 *n*-propanol:cyclohexane eluted the DITy, and *n*-propanol alone brought out the MITy. This separation was applied to a variety of preparations from rats receiving  $I^{131}$ : alkaline thyroid gland hydrolyzates, water or alkaline extracts of free amino acids of the thyroid, and plasma. When protein was present, a fast-running unknown often appeared in addition to spreading of the  $T_4$  peak. However, the authors did not appear to feel that a preliminary extraction was necessary, as is usually done by other laboratories.

Dowex-1 resin, 200-400 mesh, was equilibrated by Galton and Pitt-Rivers (1959a) with 0.2 *M* sodium acetate-acetic acid buffer at pH 5.6, and packed into a column 1 cm. in diameter and 3 cm. high. Serum or thyroid gland hydrolyzates at pH's of 7.4-9.6 were added and the protein eluted with the first one or two 3-ml. fractions of acetate buffer at pH 5.6. The pH was then lowered by increasing the proportion of acetic acid. At pH 3.6, thyroglobulin was removed. Then MITy and DITy were eluted by changing to 1.15 and 10% acetic acid, respectively, resulting in pH's of 3.0 and 2.2.  $T_3$  and  $T_4$  required 30-50% acetic acid, coming off nearly together at pH 1.8-1.4. Inorganic iodide remained tenaciously on the resin until 3 *M* NaBr solution was added. The acetic acid-water mixtures could be taken to dryness under reduced pressure and redissolved in 3:1 methanol-ammonium hydroxide solution for paper chromatography.

Galton and Pitt-Rivers (1959a) also were able to adsorb iodinated materials present in 0.9% saline extracts of homogenized kidney tissue. An increased head of pressure was required to ensure continued extraction. For liver and finally for kidney, these authors (1959b) seem to prefer homogenizing the tissues with 0.9% NaCl solution in the cold, acidifying to pH 2, and extracting 5 times with butanol in a more traditional manner.

Mandl and Block (1959) describe the use of a cellulose powder column with a solvent system of 3 volumes *sec*-butanol plus 1 volume 3% (w/v) aqueous  $NH_3$  for the separation of mixtures of  $I$ , MITy, DITy,  $T_3$ , and  $T_4$ . They also use an ingenious dye labeling technique to facilitate visual separation of major constituents. During prior extractions, about 200  $\mu$ g. each of thymol blue and phenol red are added to the volume being chromatographed. Thymol blue, as a green-yellow band, precedes

the pink phenol red through the column. The fraction starting just before the thymol blue band and ending before the phenol red contains all iodothyronines plus some other amino acids. Collection of the remaining solvent volume starting with the pink band yields I<sup>-</sup>, MITy, DITy, plus other amino acids. These authors then reduce each of these two fractions to dryness *in vacuo* and redissolve in 1 ml. of methanol-concentrated NH<sub>4</sub>OH (99:1 v/v) for subsequent paper chromatography.

An elegant procedure combining temperature control of the Dowex-1 column and continuous gradient elution with formic acid has been described in detail by Wynn *et al.* (1959). Good separation of MITy, DITy, and 3,5-T<sub>2</sub> was obtained at 45°C. with formic acid concentrations from 5 to about 25%. The temperature is then raised to 55°C. and the formic acid concentrations changed to 70–80%. Under these new conditions, T<sub>3</sub> came off just ahead of T<sub>4</sub> and subsequent paper chromatography showed adequate separation of the two. Iodide was held on the column.

An important part of the Wynn *et al.* procedure with serum is the adsorption of 88–98% of the iodoamino acids present on the resin by 1 hour's shaking of 20–50 ml. of serum with 3–5 ml. of resin suspension at a pH of 10.5–11.0. Proteins and ammonia were removed with water. After acidification and degassing, the resin and adsorbed iodoamino acids are transferred to the column for gradient elution with formic acid. The 5-ml. fractions collected could be used directly, ashed for iodine determinations, or reduced *in vacuo* for paper chromatography.

### 5. Paper Chromatography

The ease of handling minute volumes of solution (1–100  $\mu$ l.) on filter paper strips or sheets has made this technique more widely used than column chromatography.

Very early in the introduction of this technique, Taurog and Chaikoff (1948) demonstrated, using direct chromatography of butanol extracts of unhydrolyzed plasma, that T<sub>4</sub> was present as the free amino acid. Confirmed by many other workers (cf. Barker, 1955), this observation led to a fundamental re-evaluation of the nature of the circulating thyroid hormone as being the free amino acids T<sub>4</sub> and, to a much smaller extent, T<sub>3</sub>. The combination of T<sub>4</sub> and T<sub>3</sub> with specific plasma proteins has already been discussed.

In 1948 Hird and Trikojus had noted the presence on paper chromatograms of an unidentified ninhydrin-reactive spot in the alkaline hydrolysis mixture from an iodinated casein which they suggested might be 3,5,3'-triiodothyronine, at that time undescribed. Three years later, Gross and Leblond (1951) also noted an unknown radioactive compound on paper chromatograms of thyroid and plasma extracts from rats injected

with  $I^{131}$ . Gross and Pitt-Rivers (1952, 1953) and Roche and co-workers (1952) identified 3,5,3'- $T_3$ .

In 1959 Werner and Block reopened the question of the nature of the circulating PBI, on the basis of stable iodine determinations, showing about half to be present as MITy and DITy. They employed the hot butanol extraction of serum already discussed, and then paper chromatography. The discrepancy between no radioactive MITy or DITy and 50% of the stable PBI as MITy and DITy was explained on the basis of dilution of the  $I^{131}$  by the large MITy and DITy pool in the thyroid. Using a combination of preliminary Dowex-1 column separation followed by final paper chromatography, Wynn (1960) has identified the serum PBI of euthyroid and of Graves disease patients as being 96%  $T_4$  and a maximum of 4% "diiodotyrosine-like." The latter compound is not identical with DITy, however, nor is it any other of the common or rare iodo-compounds mentioned by other workers as found in serum. No MITy was found.

Kono *et al.* (1960), using an unheated butanol extract of serum processed in a way similar to that of Werner and Block except that the thiouracil originally added was omitted, have regularly found  $T_4$  and  $I^-$  but no iodotyrosines. They point out that thiouracil and its disulfide are carried through the extraction procedures and give spots on paper with  $R_f$  values corresponding to MITy and DITy. Dimitriadou *et al.* (1960) demonstrated the absence of iodide in the chromatographic spots corresponding to MITy when thiourea or thiouracil was added to the plasma prior to extraction. On the other hand, Beale and Whitehead (1960) have reported finding serum MITy and DITy in quantities comparable to Block and Werner's, with a combination of  $H^3$ -acetyl labeling and isotope dilution techniques.

Bird and Farran (1960) have introduced a preliminary extraction of 4-6 ml. of plasma with an equal volume of chloroform, repeated twice, to remove lipid material interfering with paper chromatography. A butanol extract is then chromatographed in butanol:ethanol:2  $N$   $NH_4OH$  (5:1:2) and butanol:acetic acid:water (12:3:5). In contrast to the findings of Werner and Block, radioactive MITy and DITy have been encountered after administration of  $I^{131}$  to patients, but no chemically detectable iodotyrosines. The problem of MITy and DITy in plasma must be considered unresolved at present.

Paper chromatography has been widely applied to practically every phase of thyroid biochemistry. In many instances, autoradiography of  $I^{131}$  compounds following chromatography has allowed detection of exceedingly minute amounts. Using this approach, Roche *et al.* (1955)

demonstrated formation of small amounts of 3,3'-T<sub>2</sub> and 3,3',5'-T<sub>3</sub> by the thyroid gland in addition to the previously known T<sub>4</sub> and 3,5,3'-T<sub>3</sub>. Ingenious variations and combinations of procedures have permitted identification of these substances in blood plasma (Roche *et al.*, 1959).

Metabolic products of T<sub>4</sub>, primarily tetraiodothyroacetic acid (Ac<sub>4</sub>) and T<sub>3</sub>, have been detected by procedures involving chromatography of both nonradioactive and radioactive compounds (Galton and Pitt-Rivers, 1959b). It should be pointed out that chromatographic identification can sometimes be deceptive if only one or two solvents are employed, since the *R<sub>f</sub>*'s of two compounds may well coincide. Besides T<sub>4</sub> and T<sub>3</sub> (Lassiter and Stanbury, 1958), this was noted for the acetic and propionic acid analogs and T<sub>3</sub> itself (Tata *et al.*, 1957). Conjugates of T<sub>4</sub> with glucuronic and sulfuric acids were first noted on paper (Taurog, 1955; Roche and Michel, 1960). These have been identified by the return of the radioactivity *R<sub>f</sub>* to the T<sub>4</sub> position following treatment with  $\beta$ -glucuronidase or sulfatase.

It must be remembered that it is relatively easy to overload the paper, not only with iodo-compounds, but even more so with salts or protein. When the origin is even slightly encrusted, thorough wetting with the chromatographing solvents is impossible, leading to inconsistent separation. Another important factor is the volume of solution to be applied. Many ingenious devices have been proposed to enable application of large volumes by continued evaporation on the paper in a stream of warm air. The very factor of greatly increased surface leading to rapid evaporation may result in a simultaneous accelerated oxidation of sensitive compounds. Another aspect has not been stressed—that of circular chromatography at the origin itself by repeated applications of too dilute a solution. Thus the origin becomes a "halo" rather than a compact spot a few millimeters in diameter.

These considerations together have resulted in a trend toward as much separation of iodo-compounds preliminary to paper chromatography as feasible, often on a column, plus concentration of the final solution so that the volume applied can be kept down to 10–50  $\mu$ l.

In the endeavor to separate the large number of compounds, each only slightly different from its nearest chemical relative, a wide variety of solvents has been employed. *n*-Butanol with formic or acetic acid in water is useful for separating I<sup>-</sup>, MITy, and DITy from the thyronines which migrate more rapidly. *n*-Butanol saturated with 2 *N* NH<sub>4</sub>OH will separate some of the thyronines, and increasing the concentration of ammonium hydroxide to 6 *N* or using isopentanol:6 *N* NH<sub>4</sub>OH may improve the resolution of a few specific compounds. We have not seen

TABLE II  
*R<sub>f</sub>* VALUES (X 100) OF VARIOUS IODINATED COMPOUNDS OBTAINED WITH ASCENDING CHROMATOGRAPHY  
 UNDER CONDITIONS SPECIFIED IN TEXT<sup>a</sup>

Compound	Solvent mixtures <sup>b</sup>				
	Methanol: 0.2 M ammonium acetate	Butanol: 2 N NH <sub>4</sub> OH	Butanol: 2 N HOAc	<i>tert</i> -Amyl alcohol: NH <sub>4</sub> OH	Collidine: water: ammonia
Thyroxine	19	29	88	23	55
<i>O</i> -Methyl thyroxine		53	61		
Thyroxine glucuronide		12	70		91
Thyroxamine		86	76		
3,5,3'-Triiodothyronine	27	47	75	54	
3,3',5'-Triiodothyronine		21	63	22	
3,5-Diiodothyronine	54	26	34	34	
3,3'-Diiodothyronine		27	63	36	0
Tetraiodothyropropionic acid	34	45	91	39	62
Tetraiodothyroacetic acid	31		90		
Tetraiodothyroacrylic acid	41	46	93	36	63
Tetraiodothyroformic acid	41	45	90		
3,5,3'-Triiodothyropropionic acid	45	62	88	48	79
3,3',5'-Triiodothyropropionic acid	40	21	88	60	85
3,5,3'-Triiodothyroacetic acid	46	64	88	60	85
3,5-Diiodothyroacetic acid	62	62		54	
3,3'-Diiodothyroacetic acid		47		45	
3,5-Diiodotyrosine	51	7	40		24
3-Monoiodotyrosine	60	6	32	11	
Thyronine	0	34	41		
Tyrosine	62	7	12		
Sodium iodide	77	23	14	20	74

<sup>a</sup> Based primarily upon data obtained by Dr. Nicole Etling.

<sup>b</sup> Prepared as follows: (a) Methanol 100 ml., 0.2 M ammonium acetate 250 ml. (b) *n*-Butanol 250 ml., 2 N ammonium hydroxide 250 ml., thoroughly equilibrated, completely separated, top (butanol) layer placed in chromatography jar and bottom (aqueous) layer in a small beaker. (c) *n*-Butanol 156 ml., 2 N acetic acid 44 ml., as in (b). (d) Tertiary amyl alcohol 100 ml., 80 ml. of water, 20 ml. of concentrated ammonium hydroxide, treated as in (b). (e) Collidine saturated with water, concentrated ammonium hydroxide placed in a small beaker to furnish ammonia atmosphere.

enough improvement in the performance of butanol:2 *N* NH<sub>4</sub>OH from the addition of dioxane to justify the necessary rigorous purification of this material.

Table II shows *R<sub>f</sub>* values obtained in this laboratory for a variety of iodo-compounds with five useful solvent mixtures. Because of differences in operating conditions, each laboratory should set up its own reference values. For most accurate results, reference standard spots should be run simultaneously with each set of unknowns. The values shown were obtained at 23°C. using ascending chromatography carried out in large, covered glass cylinders. No supporting frames are used, since the solutions of references and unknowns are spotted with micropipets along a line 25 mm. above one edge of a large sheet of Whatman No. 1 which is then formed into a cylinder with the line of spots at the bottom end. The two edges to be approximated are stapled in that position using a wide-opening stapling machine, making certain that the edges do not overlap. The glass jar contains a 1-cm. layer of the solvent mixture, with the solution for gas equilibration, if any, held in a small beaker in the center of the bottom. The paper cylinder will support itself even when wet, although it must not touch the side of the cylinder.

Larger quantities of material can be separated using a heavy filter paper, such as Whatman No. 3 MM, and applying the solution in a line rather than a spot, although the ends of the line should be at least 20 mm. from the sides of the paper sheet to avoid convection currents. As much as 50  $\mu$ l. of solution can be added per centimeter of starting line. A strip can be cut the entire length of the paper for identification of compounds after the run has been finished. On the basis of the trial strip, an entire horizontal band can be cut out of the dried chromatogram and eluted for further evaluation or as a preparative technique. Complete testing of each type of paper with all compounds being separated is necessary. Even though the same solvent mixture may be employed, the different characteristics of each paper make this control imperative.

Techniques for the study of chromatograms will be discussed in a later section.

## B. PEPTIDE-LINKED IODOAMINO ACIDS

### 1. Isolation

The principal source of iodoprotein is, of course, the thyroid gland with its thyroglobulin. Traditionally, this material is extracted with saline from sliced or macerated thyroid tissue. Separation of a single component by salting out with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at low temperatures leads to isolation of the "thyroglobulin," but there is some question about the physio-

logical aspects of being so arbitrary about discarding even minor proteins. There may well be justification for the use of the entire thyroid gland, as is done in many studies on  $I^{131}$  incorporation.

Rall *et al.* (1960) have shown that the most consistent method of isolating a uniform species of thyroglobulin was by differential high-speed centrifugation, rather than by salting out. From this study, they define thyroglobulin as a 19-S globular protein. The same workers have prepared iodoproteins from an  $I^{131}$ -accumulating rat tumor by extraction with 0.9% NaCl. In addition to 19-S sedimenting thyroglobulin, they found a 4-S iodoprotein with an electrophoretic pattern showing three peaks of radioactivity, two of which overlapped the thyroglobulin. Iodo-amino analysis showed a disproportionate amount of MITy, suggesting slower and incomplete iodination of this 4-S protein. The serum of these animals contained a similar iodoprotein, probably released by the tumor. This may be related to an abnormal serum iodoprotein moving electrophoretically as albumin encountered in patients with goiter, thyroid carcinoma, or other thyroid disease. An insoluble iodoprotein associated with rat tumor subcellular particles was found by DeGroot *et al.* (1958) to require strong alkali or proteolysis for solubilization.

Rall *et al.* (1960) have also summarized much of the current information about unusual protein synthesis by thyroid tumors and congenital defects of the gland. Most of these abnormal situations are first detected by discrepancies between metabolic level of the patient and his PBI level,  $I^{131}$  incorporation, or other evidence of thyroid function. Electrophoresis, chromatography, or butanol extractibility then reveals more about the existence of an abnormal material and may assist in its isolation.

## 2. Protein Hydrolysis

If the various iodoamino acids, peptide linked, are to be further characterized, hydrolysis of the covalent bonds is necessary rather than incineration, as for complete liberation of the iodine as  $I^-$ . The classic procedure for this, following Harington (1926) and Blau (1933, 1935), is to heat 100 mg. of tissue with 1 ml. of 2 N NaOH or 1 ml. of saturated  $Ba(OH)_2$  in a boiling water bath for 16 hours (Roche *et al.*, 1954). Some destruction occurs, even with the barium hydroxide (Taurog *et al.*, 1950), but the extent of this cannot be completely evaluated, due to uneven destruction of added  $T_4$  and to a lack of adequate methods for chemical assay of  $T_4$  while actually in protein combination. This loss is certainly far less during alkaline hydrolysis than during acid; in fact, heating of any  $T_4$  derivative in acid will result in considerable decomposition and is to be avoided.

Many investigators prefer enzymatic hydrolysis, in spite of the greater



time involved and a less complete hydrolysis of protein. Roche *et al.* (1954) propose a 3-day incubation at 38° of 100 mg. of tissue suspended at pH 8.5 with Armour's unfractionated trypsin being added at the start and renewed after 1 and 2 days. Mandl and Block (1959) prefer Viobin's pancreatin for 3 days, followed by crepsin for an additional 2 days. No one appears to have tried the continuous-extraction type of process widely employed during urinary steroid hydrolysis. With either alkaline or enzymatic hydrolysis, the reaction might be speeded up by continuous removal of the free amino acids, although conditions are not especially favorable for extraction at a pH such as 8.5.

Artificially iodinated proteins, following the early discovery of Ludwig and von Mutzenbecher (1939), have posed a special problem of analysis. The usual methods of hydrolysis and extraction yield considerably more "thyroxine-iodine" than indicated by bioassay. Reineke and Turner (1945) arbitrarily increased the Ba(OH)<sub>2</sub> concentration to 40%, thereby obtaining chemical results agreeing more closely to the metabolic. It is not clear whether such discrepancies are due to a great excess of MITy and DITy or to iodohistidine and other nonhormonal forms of organic iodine.

### 3. Separation of Liberated Iodo-Compounds

The large amount of salt or other extraneous material present after hydrolysis with either base or enzymes makes some sort of preliminary extraction obligatory. Although Block *et al.* (1958) extract the dried hydrolysis mixture with 99:1 methanol-NH<sub>4</sub>OH, they also make use of a more conventional procedure by acidifying to a pH range of 3-4 and extracting repeatedly with *n*-butanol (Mandl and Block, 1959). These combined solutions are taken to dryness *in vacuo* and dissolved in a small volume of solvent mixture appropriate to the next step.

Substances present in the extract can then be separated by column or paper chromatography, following procedures earlier discussed.

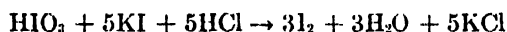
## III. Chemical Methods of Determination

It was mentioned in the Introduction that the actual methods of determining specific iodo-compounds were so nonspecific in themselves as to require combination with some preliminary separation procedure. It will be evident from the material presented in this section on technique that the determination of iodide is by far the most sensitive, but is obviously the least characteristic.

## A. IODINE

1. *Macromethods*

The 1914 procedure of Kendall, based on the earlier work of Hunter, was applicable only to thyroid material in order to have adequate quantities of iodine. Organic matter was incinerated with NaOH plus KNO<sub>3</sub> for oxidizing purposes. The organically bound iodine was changed to iodide plus iodate, any of the former remaining then being oxidized to the latter with bromine. The iodate was reacted with an excess of iodide in acid solution to liberate free iodine



which was then titrated with standard thiosulfate in the conventional manner.

Many attempts have been made to improve the sensitivity of the Hunter-Kendall method of alkaline incineration, oxidation to iodate, and conversion to I<sub>2</sub> so that it could be applied to material of lower iodine content than thyroid, especially blood serum. Probably the most ingenious was the proposal by Michel and Lafon (1945) to extract the I<sub>2</sub> into CS<sub>2</sub>, then reduce the iodide, reoxidize to iodate, and put it through the iodide reaction again to yield a "two-stage amplification." Others introduced microtitration with 0.001 *N* thiosulfate, direct colorimetry using the blue starch-iodine reaction, and other modifications (summarized by Pitt-Rivers, 1950). A variety of difficulties in each case led to inconsistencies.

2. *Micromethod, Based on Sandell and Kolthoff (1937)*

Nearly all procedures being used today are based on this early demonstration of iodide catalysis of arsenite reduction of ceric ions. Since the reaction involves a change from yellow ceric to colorless cerous proceeding at a rate proportional to iodide concentration with quantities of the order of 0.005 to 1.0 μg., the decrease of optical density must be followed at standard time intervals. Among ions, the catalysis is highly specific for iodine, osmium being the only similar accelerator. It should be noted that mercury and silver will interfere with iodide catalysis, probably by precipitating iodide, and chloride will potentiate the iodide catalysis, although not reacting itself.

A surprising discovery was made by Bowden *et al.* (1955), that DITY, T<sub>4</sub>, and similar organic iodine compounds would catalyze the arsenite-ceric reaction on filter paper. Further study revealed that all phenols with ortho-situated iodine, plus 3,5-diiodothyronine, liberated free iodine on treatment with ceric sulfate, as did KI. Except that no iodine was found

after ceric sulfate treatment of iodate, this would implicate free iodine as the catalyst, as has been suggested by Dubravčić (1955) in his formulation of the two steps of iodide catalysis of the arsenite-ceric reaction



Recycling of the iodide ion would explain the tremendous sensitivity of its catalysis in this reaction. Dubravčić stresses the importance of chloride on the basis of removing some inhibitors such as Hg or Ag, but especially as changing the  $E_h$  of the reaction mixture to prevent iodate formation



or even to reverse any iodate already present



Morreale de Escobar and Gutiérrez Ríos (1958) indicate that chloride is necessary for the establishment of first-order reaction kinetics.

The convenience of at least qualitative direct location of organic iodine compounds on chromatograms has led to widespread adoption of the Bowden *et al.* procedure. This is best carried out by placing on a clean glass plate a piece of the same paper as that used for the chromatogram, cut to be a few millimeters larger in both directions than the latter. A fresh mixture of equal parts of 10% ceric sulfate and 5% arsenious acid, both in 1 *N*  $\text{H}_2\text{SO}_4$ , is then added to soak the blank paper evenly. The dried chromatogram is carefully laid on, starting at one edge to avoid trapping air or wrinkling the sheet. Another glass plate is placed on top and pressure exerted to obtain even wetting. The papers are left in contact for 10–30 minutes, depending on the size and proximity of spots of decolorization. The top plate is then lifted off from one edge as is the chromatogram. This should be dried rapidly in a stream of warm (but not hot) air to stop fading of the background. The necessary measurements must be made within a day because of disintegration of the paper. Manipulations with the solutions and paper are best done on a stainless steel bench top to facilitate thorough removal of the highly toxic and corrosive solutions.

Useful modifications of the Bowden procedure have been introduced for convenience in handling, higher contrast between spots, and neutralization of the acid. Mandl and Block (1959) spray both sides of the dried chromatogram with a fresh mixture of 2 parts 10% ceric sulfate in 10%  $\text{H}_2\text{SO}_4$  plus 3 parts 5%  $\text{NaAsO}_2$  in water, using about 50 ml. of reagent for an 18- × 20-in. sheet of Whatman No. 3. The sheet is partially dried for about 5 minutes, and then both sides are sprayed with

0.05% methylene blue in water. When the reaction has proceeded sufficiently, the paper is exposed to ammonia vapor to neutralize the sulfuric acid; this is indicated by a change in the background from light blue to bright yellow. The iodo spots are blue.

As pointed out by Wynn (1960), if this is to be carried out on a chromatogram developed in collidine before use of the ceric-arsenite spray, the dried paper must be hung in an acetic acid atmosphere to neutralize a basic residue left by the collidine. It must again be emphasized that spraying the dangerous arsenite-ceric reagent should be done with great care. A water-rinsed cabinet, such as the one described by Fiebig and Siegel (1958), inside a well-ventilated hood is useful. We have found that this procedure requires a thick paper for success; Whatman No. 1 does not seem as satisfactory.

A follow-up to the original Bowden *et al.* procedure also heightens contrast of the spots and preserves the chromatograms (Bird and Farran, 1960). As modified by Kono *et al.* (1960), the paper is removed from the glass plates and dried, then it is dipped in a fresh 3% aniline solution in acetone. The spots remain white, but the background turns green. After drying, the paper is dipped in 5% sodium carbonate or bicarbonate in water and dried. The background becomes dark blue. Despite some spreading of the spots, permanence of the chromatograms is achieved by neutralization of the acid.

There have been several reports of other attempts to improve contrast by adding a variety of reagents, such as brucine sulfate, sulfanilic acid (Gawienowski, 1957), *o*-phenylenediamine (Fletcher and Stanley, 1956), fluorescein, and anthranilic acid (Stolc, 1958). Introduction of any such toxic substance as brucine does not seem justified by the results, and none of the others has been as successful in this laboratory as the methylene blue, followed by alkalinization. An especially intriguing reagent employs ferroin, as proposed by Dragúnová and Langer (1956). This is prepared by dissolving 1.485 gm. *o*-phenanthroline monohydrate in 100 ml. of water containing 685 mg. of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ . The original report suggests addition of 0.5 volume of this reagent to 1 volume of the ceric-arsenite mixture proposed by Bowden *et al.* to obtain red spots on a blue background. Our experience has been that this addition does not enhance the sensitivity of the reaction and that the pink spots disappear rapidly upon removal of the top glass plate. Robbins (personal communication) has modified the combination of reagents to provide a considerable excess of arsenite by mixing 6 parts of 5% sodium arsenite, 1 part ceric sulfate, and 1 part ferroin reagent.

Gmelin and Virtanen (1959) propose the use of a mixed ferric chloride-ferriicyanide-arsenite reagent as a spray. Three stock solutions are pre-

pared and kept dark: (a) 2.7 gm.  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 100 ml. 2N HCl; (b) 3.5 gm.  $\text{K}_3\text{Fe}(\text{CN})_6$  in 100 ml. water; (c) 5 gm.  $\text{NaAsO}_2$  dissolved in 30 ml. of cold 1 N NaOH and 65 ml. 2 N HCl added with good stirring. Immediately before use, a mixture is made of 5.5 and 1 parts, respectively. The paper is sprayed from both sides and put without pressure between two glass plates. After 15 minutes, the paper is thoroughly washed with distilled water and dried. As ferrocyanide is formed, it immediately is removed by precipitation as Prussian blue. The  $\text{I}_2$  is reduced to  $\text{I}^-$  by the arsenite and thus can recycle. The authors claim a sensitivity about 10 times that of the ceric-arsenite reaction, but our experience has not yielded any more success than when using methylene blue. Furthermore, the procedure must be carried out in the dark to avoid complete discoloration of the paper.

Kono *et al.* (1960) have recently pointed out "false reactions" from chloride ions, propylthiouracil, thiouracil and its disulfide, as well as from methionine, cystine, and tyrosine. Tryptophan gave a reddish color, but from the  $R_f$ 's shown, the others clearly could be confused with iodotyrosines in some solvent systems. As already mentioned, these authors suggest that the large quantities of nonradioactive MITy and DITy reported present by Block *et al.* (1960) in the eluates from paper chromatograms of serum may have been carry-over of thiouracil added to the original serum.

Kono *et al.* (1960) comment, "a great many organic compounds when used in large amounts gave rise to white spots by directly reducing ceric ion." In this laboratory, we have had occasion to encounter this trouble from citrate present in a concentration of 0.05 M. Considerable re-examination is necessary of previous findings "establishing" the presence of various iodinated compounds in biological material. Attention may again be called to the frequent chromatographic encounters with " $\text{T}_3$ " in the days before realization that relatively large quantities of the acetic acid analog of  $\text{T}_4$  could be produced metabolically by various peripheral tissues.

*Quantitative application of arsenite-ceric sulfate reaction.* Technical difficulties prevent effective quantitation with the Sandell-Kolthoff reaction on paper. Mandl and Block (1959) described a method of eluting the iodoamino acid from a portion of the paper chromatogram using 1 N  $\text{H}_2\text{SO}_4$ -arsenious acid, preparing an extract ready for direct colorimetric determination of iodine. Quantitative studies showed the remarkable situation that MITy, DITy, 3,5-diiodothyronine ( $\text{T}_2$ ),  $\text{T}_3$ , and  $\text{T}_4$  all reacted at 31.3% of the decolorization catalysis rate of  $\text{I}^-$ . The same figure was obtained for  $\text{T}_4$  by Wynn (1960), but the study of Morreale de Escobar and Gutiérrez Ríos (1958) placed emphasis on

iodine ortho to a phenol, as did that of Bowden *et al.* (1955). DITY was as active a catalyst as  $T_4$ , but  $T_3$  was less, and 3,5- $T_2$  least of all, in contradiction of Mandl and Block.

Thus, a direct estimation of organic iodine not only loses 69% sensitivity, but is susceptible to the many types of errors discussed above. It is highly desirable that all organic materials should be incinerated so that all iodine is present as  $I^-$ . The Schöniger (1955) procedure for ignition of organic samples in a closed flask containing an oxygen atmosphere seems well adapted for quantitative work following paper chromatography. The paper itself could serve to support combustion in the platinum holder. Mandl and Block (personal communication) have used it to quantitate iodine in segments cut out of chromatograms. If  $SO_2$  from sulfur-containing amino acids can be completely eliminated, possibly by oxidation to sulfate (Boëtius *et al.*, 1958), this should prove a highly useful and simple technique.

For the ceric-arsenite reaction, many different combinations of reagents and reaction conditions have been used (surveyed by Foss *et al.*, 1960).<sup>3</sup> Since this procedure makes use of acceleration of a reaction rate, involving gradual disappearance of color, it is obvious that all conditions must be scrupulously controlled, especially temperature. This can be adjusted up or down to achieve more rapid or more delayed reaction rates, respectively, but must be held within a few hundredths of a degree at the temperature selected. It is usually carried out at 20°–40°C.

For routine purposes, it is adequate to make a colorimeter reading at a single time. This assumes linear decolorization, and many workers prefer two readings, plotting both of them. An elegant modification is to use a recording colorimeter (Chaney, 1950; Mandl and Block, 1959) to obtain a continuous record covering the 20–70% transmission range. In this case, the room temperature is the reaction temperature and must be carefully regulated. For reference, standards covering the entire range

<sup>3</sup> Since Astwood's modifications of the Zak procedure have not been published in full, the final colorimetric step will be given here to complete the description started earlier (Section II, A, 2). To the 0.5 ml. remaining in each digestion tube add 10.0 ml. of water and 2.0 ml. of arsenious acid solution. Mix and bring to 32°C. At timed intervals, quantitatively pipet in 0.50 ml. of ceric ammonium sulfate solution, mix rapidly, and continue the incubation for exactly 30 minutes. Read in an appropriate colorimeter with a blue filter or in a spectrophotometer at 420 m $\mu$ ; enough time must elapse between each addition of ceric reagent to allow the necessary 30-minute colorimeter reading. The arsenious reagent consists of 3.0 gm. of  $As_2O_3$  and 87.5 gm. of NaCl dissolved in 200 ml. of 1% NaOH; after mixing 764 ml. of  $H_2SO_4$  and 1 liter of water, cool and add to the above. For ceric ammonium sulfate, dissolve 24 gm. in 1 liter of 3.5 N  $H_2SO_4$ . The procedure has a range of 0.02 to 0.06  $\mu g.$  iodine.

of unknown values should be run simultaneously, in solutions completely duplicating all quantities of reagents present in the unknown.

Attempts have been made to arrest the arsenite reduction of cerate at a desired time in order to obtain stable colorimeter readings. Grossmann and Grosse mann (1955) put the tubes in an ice bath after 15 minutes at 37° and then add a solution of brucine sulfate. This has been criticized by Magee and Spitzzy (1959) on the double basis that cooling did not stop the reaction and that brucine merely added undesirable complications. Rogina and Dubravčić (1953) stop the ceric-arsenite reaction by adding an excess of ferrous ion (as ferrous ammonium sulfate) which completely reduces all remaining  $Ce^{IV}$ ; ferric ions (equivalent to the ceric previously remaining) are then determined as the red ferric thiocyanate.

### B. AMINO ACID FUNCTIONS

The usual ninhydrin reaction with  $\alpha$ -amino-carboxylic acids takes place as indicated in Fig. 2. The final purple color is similar for most

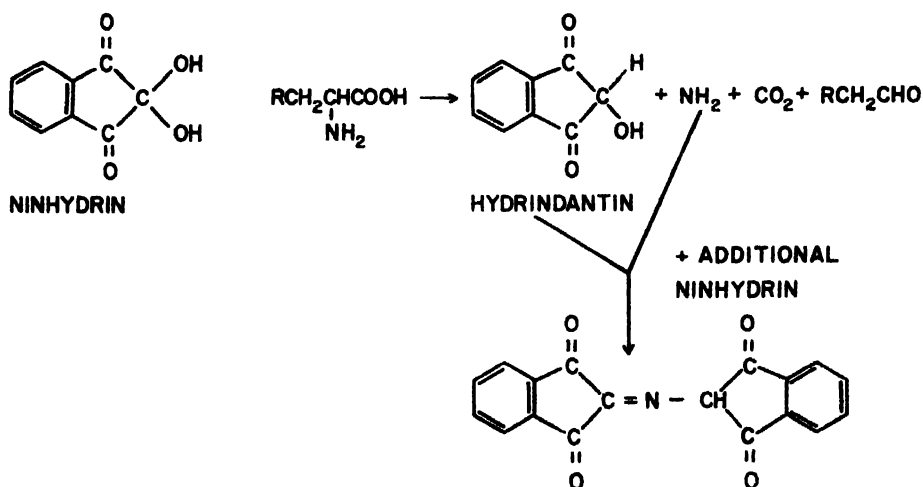


FIG. 2. The ninhydrin reaction.

amino acids, but there are variations, suggesting that the above formulation is not complete. The iodothyronines,  $T_2$ ,  $T_3$ , and  $T_4$ , yield a blue-gray color, and 25–50  $\mu g.$  are required, about 5–10 times the amounts of simpler amino acids. Only part of this difference can be ascribed to the greater molecular weight of the iododiphenyl ether. MITy and DITy are detectable in somewhat smaller quantities.

The most sensitive and reproducible procedure is to spray the paper

chromatogram with 0.2% ninhydrin in *n*-butanol saturated with 2 *N* acetic acid. The full color is then brought out by heating in a stream of 90°C. air.

### C. PHENOLIC FUNCTIONS

Kendall and Osterberg in 1919 described a yellow color when  $T_4$  was reacted with nitrous acid in acid-alcohol, which turned red with addition of ammonia. Morton and Chaikoff (1943) developed this for quantitation of  $T_4$  and DITY in alcoholic solution. To 5 ml. of solution is added 0.2 ml. of 6 *N* HCl plus 0.5 ml. of 1%  $\text{NaNO}_2$  solution. The mixture is brought to the boiling point by a short immersion in a boiling water bath, cooled, and 0.5 ml. of concentrated  $\text{NH}_4\text{OH}$  added. Other variations of the Kendall-Osterberg reaction have not materially heightened its sensitivity, but it is occasionally valuable because of its simplicity. Roche and Michel (1947) have eliminated the heating in a modification adapted to the determination of  $T_4$  and DITY in the appropriate washed butanol extracts of hydrolyzed thyroid glands and Roche *et al.* (1947) used this method to follow production of synthetic iodoproteins. MITy could also be estimated with a special application of Millon's reagent, although Shaw (1953) encountered troublesome turbidity. Adamson *et al.* (1952) evaluated a variety of procedures adapted to determination of thyroxine in thyroid tablets and concluded that a slightly modified Roche and Michel (1947) colorimetric analysis was the most suitable.

In the early separation of both synthetic and natural  $T_3$  and  $T_4$  by column chromatography, Gross and Pitt-Rivers (1953, p. 648) added 3 ml. of 95% ethanol to 1 ml. of the  $\text{CHCl}_3$ -butanol fractions, 0.1 ml. of 11 *N* HCl, and 0.5 ml. of fresh 5%  $\text{NaNO}_2$ . The tubes were heated 30 seconds at 100°, then left at room temperature for 10 minutes. The presence of  $T_4$  or  $T_3$  was indicated by a yellow color, changing to a brownish-orange after addition of 0.1 ml. of 40% NaOH. The absorption curves, measured at 465  $m\mu$ , were nearly the same for both  $T_1$  and  $T_3$ . The color is probably due to an equilibrium as indicated in Fig. 3.

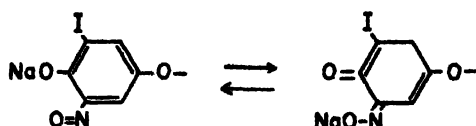


FIG. 3. See text.

Following up Kendall and Osterberg's reaction after Harington had proven the structure of  $T_4$ , Komant (1930) discovered that Pauly's



reagent, diazotized sulfanilic acid, reacted with  $T_4$  and DITy to give a red or purple color. The corresponding tetrabromothyronine, dibromotyrosine, and dichlorotyrosine did not react. Moser (1947) added 4 ml. of 10%  $Na_2CO_3$  solution to 5 ml. of  $T_4$  solution, cooled to  $0^\circ$  and mixed with 4 ml. of freshly prepared diazobenzenesulfonic acid also at  $0^\circ$ . Four minutes after the start of color development, 2 ml. of 3 *N* NaOH were added and the red color measured at 510  $m\mu$ .

The reaction involved probably splits off one iodine ortho to the 4'-hydroxy (Barac and Morren, 1955) (Fig. 4). In alkali, this product then probably forms a quinoid similar to that shown in the figure.

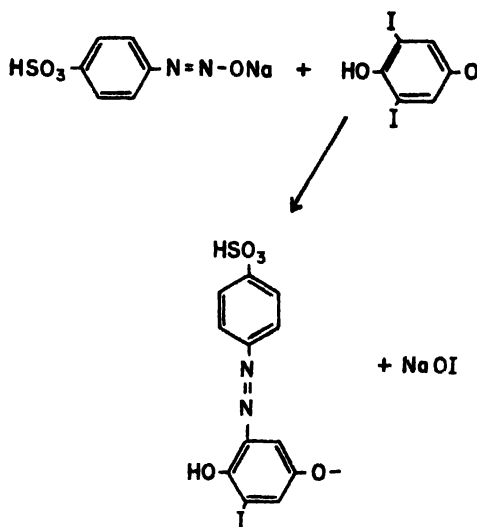


FIG. 4. Probable reaction of  $T_4$  and DITy with Pauly's reagent.

After a thorough investigation of the reaction between a series of 29 diazotized amines and  $T_4$  in aqueous alkaline solution, Winikoff and Trikojus (1948) selected  $N'$ ,  $N'$ -diethylsulfanilamide as the preferred reagent. From a stock solution of 1.14 gm. dissolved in 9 ml. of 10 *N* HCl and made to 100 ml. with water, 5 ml. are diazotized at  $0^\circ C.$  with 5 ml. of 4.5%  $NaNO_2$ . Twenty milliliters of ice-cold water are added and the mixture left for 10 minutes. To 2 ml. of  $T_4$  solution containing 10 to 50  $\mu g.$  per milliliter of 0.1 *N* NaOH in a glass-stoppered 15-ml. centrifuge tube are added 2 ml. of *n*-butanol and 1 ml. of 2 *N* NaOH. After mixing these, one adds 3 ml. containing 1 ml. of 2 *N* NaOH plus 2 ml. of the final diazotized sulfanilamide solution described above. The tube is stoppered, thoroughly shaken, and then gently agitated for 24 hours at  $25^\circ$ . After separation of the layers by centrifugation, 0.75 ml. of the butanol layer is added to 0.25 ml. of 1 *N* NaOH in ethanol, mixed, and

read at 540  $m\mu$ . The calibration curve is linear from 10 to 50  $\mu\text{g.}$  per milliliter. Although not sufficiently sensitive for direct analysis of  $T_4$  at physiological concentrations, the butanol extraction enabled separation of the  $T_4$  color from other materials, such as DITy. The quantitative aspects of this procedure do not seem to have been evaluated with  $T_3$  since its discovery, but the reaction proceeds smoothly.

Many different procedures have been proposed for diazotization of spots on paper. The one found most convenient in this laboratory makes use of stock solutions of 5%  $\text{NaNO}_2$  and 1% sulfanilic acid (dissolved in water, 9 ml. concentrated  $\text{HCl}$  added and diluted to 100 ml.) kept in the cold. Equal parts of these stocks are mixed a few minutes prior to use; the mixture need not be kept cold. The dried chromatogram is sprayed with 5%  $\text{Na}_2\text{CO}_3$  and allowed to become nearly dry. The diazonium solution is then sprayed on and the spots rapidly become apparent, purple in the case of  $T_4$  and  $T_3$ , red-orange for iodotyrosines. Some workers spray in the reverse order or with a mixture of diazonium solution and  $\text{Na}_2\text{CO}_3$ . The background is usually much darker, especially with the latter modification.

Trials have been made of other coupling reactions of ortho-substituted phenols (Barker, unpublished), including the diethylsulfanilamide of Winikoff and Trikojus and chloroquinone imines, but nothing superior to diazotized sulfanilic acid has been found for use on paper.

#### IV. Physical Methods of Determination

##### A. ULTRAVIOLET SPECTROPHOTOMETRY

As far back as 1936, Heidt studied the ultraviolet (UV) absorption spectra of  $T_4$  and related compounds, but concluded that differences were insufficient for simultaneous differentiation. Ginsel later (1939) compared absorption spectra of thyroglobulin,  $T_4$ , and DITy at acid and alkaline pH's and evolved a differential extinction coefficient representing the difference between the latter and the former. He reported that, using reference curves for DITy and  $T_4$ , thyroglobulin contained two molecules of DITy to one of  $T_4$ .

Marenzi and Villalonga (1941) discovered that iodination of phenols (normally in the ortho position) shifted the UV absorption maxima toward longer wavelengths and increased the extinction coefficient difference between maximum and minimum. Their observation that iodination of casein similarly shifted its absorption was extended by Reineke and Turner (1942, p. 63) to a correlation with thyroid-like activity as iodination progressed. The difficulties of applying such measurements

were illustrated by the fact that the highest UV absorption intensities were obtained on protein excessively iodinated to the point of decreased biological activity. Large amounts of iodoprotein with low thyroidal activity were obtained when  $I_2$  in propylene glycol was injected into animals (Barker and Lipner, 1949), and may represent a similar non-specific iodination reaction.

Besides confirming the shift in UV absorption characteristics of tyrosine, thyronine, and iodothyronines as iodines were added, Gemmill (1956) showed chromatographic evidence of formation of the specific compounds. He also reported that iodination of casein and insulin and even further addition of iodine to thyroglobulin produced UV changes suggestive of iodination of tyrosines. No studies were made of the biological activity, and the question of further progress to iodothyronines was not considered. Gemmill (1953) used spectrophotometric analysis to show a marked change in UV absorption of  $T_4$  upon the addition of copper acetate. The effect was primarily a considerable elevation in the minimum ordinarily seen at 290  $m\mu$  plus a slight elevation in the maximum which also was shifted from 325 to 315  $m\mu$ . This was interpreted as complex formation between copper and at least the hydroxyl group of  $T_4$ , although one might suspect involvement of the carboxyl as well.

Tata (1959) has recently renewed the interest in UV spectrophotometry of  $T_4$  by his report of a maximum at 230  $m\mu$  with an extinction coefficient in alkali about 5 times that at the conventional 325  $m\mu$ . A similar maximum at 227 was found for 3,5,3'- $T_3$ ; in this case, extinction was 8 times that at 318  $m\mu$ . Similar results were reported in 1952 by Adamson *et al.*, who deprecated the finding because DITy, 3,5-diiodothyronine, and  $T_4$  all displayed maximal absorption at 227  $m\mu$ . Further exploration of wavelengths below 260  $m\mu$  (Gemmill, 1959) has revealed that, at their individual peaks in the 210–240- $m\mu$  region, molar absorbancy indices for thyronine, 3- $T_1$ , 3,5- $T_2$ ,  $T_3$ , and  $T_4$  rise from 14,595 for the first to 49,167 and 48,561 for the last two, in that order. Halogenation of tyrosine also produced absorbance in this region, but to a less marked extent. Gemmill pointed out that this region of UV absorbance is usually related to the amino acid portion of the molecule but that the formic, acetic, and propionic acid side-chain analogs show peaks similar to  $T_4$ . Obviously, there must be further exploration of this peak and any possible response to the addition of copper.

#### B. POLAROGRAPHY

Thyroxine, 1 mg. per milliliter in aqueous ethanolic sodium carbonate solution containing tetramethylammonium iodide, was found reducible

at the dropping mercury cathode (Simpson and Traill, 1946). Three waves were obtained with half-wave potentials of  $-1.2$ ,  $-1.4$ , and  $-1.7$  volts. Since DITy yielded only the last two waves, there was sufficient difference to enable assay of  $\text{Ba}(\text{OH})_2$  hydrolyzates of iodoproteins (Simpson *et al.*, 1947). The fundamental aspects of this work were very closely confirmed a few years later by Borrows *et al.* (1949) who evolved procedures for analysis of mixtures of thyroxine and diiodotyrosine, with the latter in an excess of tenfold or greater. A disturbing feature of this work was that it revealed a considerably greater butanol extraction of DITy than had previously been reported by Leland and Foster (1932). Borrows *et al.* pointed out that this would have a considerable effect on chemical determinations, but not on the polarographic.

The problem of chemical determination of specific compounds in desiccated thyroid is still not completely resolved. Some of its aspects were discussed by Borrows *et al.* in 1949, leading up to their proposal of a polarographic analysis. Since that time, the discovery of  $\text{T}_3$  and its greater biological activity has added an unforeseen complication to what was previously considered primarily a problem of separating out the inactive iodotyrosine prior to final analysis. Stasilli and Kroc (1956) in considering the generally recognized greater metabolic effect of porcine over bovine thyroglobulin were unable to find enough evidence to decide whether this difference could be accounted for by the estimated content of  $\text{T}_3$ . Since there is now general agreement that  $3,3'\text{-T}_2$  and  $3,3',5'\text{-T}_3$  are not metabolism stimulating, the small amounts of these iodothyronines should not be a confusing element in desiccated thyroid assay.

### C. RADIOACTIVITY

By far the most sensitive technique for detecting any of these compounds is radioactive tracer labeling. Although metabolism of  $\text{C}^{14}$ -labeled  $\text{T}_4$  has been followed by Klitgaard *et al.* (1953), the difficulties are greater than with  $\text{I}^{131}$  and the latter has been employed almost exclusively. If *in vivo* labeling is desired,  $\text{I}^{131}$  administered as sodium iodide is the only practical isotope, because of the highly specific nature of thyroid gland function (Fink *et al.*, 1947). Whitehead and Beale (1959) have achieved *in vitro* marking by reacting the plasma thyroxine with tritiated acetic anhydride. Quantitation was achieved by including a standard amount of  $\text{I}^{131}\text{-T}_4$  before acetylation or of  $\text{N-(C}^{14}\text{-acetyl)-T}_4$  afterwards. Beale and Whitehead (1960) have employed this type of procedure to determine apparent amounts of  $\text{T}_4$ , DITy, and MITy present in human plasma. Since extraction procedures were not sufficiently

specific for iodothyronines or iodotyrosines, acetylation of other amino groups would constitute a potential error, as would even traces of unreacted  $H^3$ -acetic anhydride. Much further exploration will be needed before this procedure can be evaluated completely.

Paper chromatographic or electrophoretic separation is usually employed to separate the fractions of radioactivity, which may be qualitatively identified by reference spots of nonlabeled standard materials. Before staining, the chromatogram may be scanned rapidly with a sensitive survey meter to locate the spots or a record may be obtained by means of autoradiography. To accomplish this, the thoroughly dried chromatogram is placed on the emulsion side of a sheet of X-ray film, such as Kodak No-Screen X-ray. Firm contact is ensured by use of a film cassette or heavy cardboard sheets held in clamps. These manipulations should all be done in a dark room.

The light-protected film and paper combination is then left away from any source of extraneous radiation for 1 to 2 weeks to allow adequate exposure. A preliminary survey can be valuable in determining time of exposure. The film is subsequently removed and developed with the usual precautions. This procedure is especially valuable in detecting spots of weak radiation provided they are not close to strong spots. Often radioautography will show up two spots in an area yielding only one peak when quantitatively counted because of overlap.

Quantitative counting was early done by carefully cutting the entire chromatogram or electrophoretogram into pieces suited to the geometry of the detector being used (cf. Deiss *et al.*, 1952). Almost as laborious a technique, but one leaving the chromatogram intact for later color-developing processes, was a quantitative scanning of the paper along a grid (Etling and Barker, 1959). The paper can be mounted on a metal plate bearing a scale and is moved in discrete steps of 2.5–5.0 mm. under a window cut in the aluminum and lead sheets which otherwise shield the counter (Roche *et al.*, 1954). A thin end-window Geiger-Müller tube or a scintillation counter can be used as the detector. This procedure is also tedious, but does permit complete scanning even of a two-dimensional chromatogram. If the survey is carried out with great care and control spots applied for counting the starting amount of activity, it is possible to account quantitatively as well as qualitatively for the  $T_4$  and other  $I^{131}$ -labeled materials being metabolized even at levels far too low to be detected by chemical methods (Ford *et al.*, 1957).

In the past few years, several companies have made available automatic scanning equipment by which means a continuous record of a rate counting meter is obtained paralleling the strip of chromatogram.

Reasonably accurate duplication with standard solutions of the size of radioactive areas being surveyed is necessary for calibration purposes. A two-dimensional paper must be cut into suitable strips.

It is possible, of course, to elute specific areas of radioactivity for further identification, but no advantage is obtained, except for preparative purposes. In this case, a heavy paper is used, such as Whatman No. 3 MM, and the material applied along a line, as described earlier. Methods for extraction and separation described earlier yield varying amounts of solution which can be counted as such in well counters or appropriate aliquots dried in planchettes for dry counting.

## V. Summary

It is clear that the variegated chemical functions present in structure of thyroxine—a tetraiodinated, phenolic, diphenyl ether-linked  $\alpha$ -amino acid—have given rise to a correspondingly complex array of chemical procedures for the determination of thyroxine and its relatives. Although some of these present certain aspects of specificity, as the Kendall-Osterberg nitrosation or the Komant diazonium salt reaction, none really is. Furthermore, the most sensitive of all, determination of iodine at a fraction of a microgram, is the least specific of all.

It has been emphasized that separation techniques are thus of primary importance in any specific analytical procedure. Early dependence upon liquid-liquid butanol extraction has given way to paper or column chromatographic separation, most often employing butanol as moving phase.

Of several physical techniques of analysis of compounds related to  $T_4$ , radioactivity labeling is by far the most often employed, because of its extreme sensitivity. The recent discovery of a previously unmentioned absorption peak in the low UV range gives promise of valuable future applications.

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# AUTHOR INDEX

Numbers in *italic* indicate the page on which the complete reference is listed.

## A

- Abelson, D., 111, *134*, 252, *257*, 278, 299, *331*  
 Adamson, D. C. M., *376*, *382*  
 Adesman, J., 203, *260*  
 Adlercreutz, H., Jr., 44, *47*  
 Aitken, E. H., 5, 6, 7, 9, 16, 20, 23, 24, 26, 28, 29, 33, 34, 35, 37, 38, 39, 40, 41, 42, 45, *47*, *49*, *50*  
 Alba, R. T., 341, *348*  
 Albert, A., 272, 274, 290, 296, *334*, *335*, 361, *383*  
 Albright, E. C., 381, *383*  
 Allen, W. M., 19, *47*, 83, *88*, 92, 93, 97, 101, 103, 106, 112, *134*, *136*, 148, 160, *167*, 228, 234, 243, 245, *257*  
 Alpers, J. B., *356*, *382*  
 Altrock, J. R., 234, *257*  
 Angell, E., 343, *347*  
 Anner, G., 291, *335*  
 Anton, H. V., 85, *90*  
 Antoniadou, H. N., 44, 45, *50*  
 Appleby, J. I., 211, 241, 242, 243, 244, *257*  
 Aranow, L., 346, *347*  
 Archibald, R. M., 202, *257*  
 Astwood, E. B., 148, *167*, 357, 359, 360, 364, *372*, *373*, *382*, *384*  
 Astwood, S. M., 357, 359, *382*  
 Atherden, S. M., 205, *261*  
 Auerbach, M. E., 343, *347*  
 August, J. T., 302, 316, *336*  
 Avivi, P., 282, 283, 314, *331*  
 Axelrad, B. J., 274, 304, 306, *331*  
 Axelrod, L. R., 11, 19, *47*, 111, *134*  
 Ayres, P. J., 116, *134*, 205, 252, 254, *257*, 273, 278, 279, 280, 292, 297, 302, 303, 304, 305, 308, 311, 313, *322*, *328*, *331*
- ## B
- Baggett, B., 3, 15, 27, *49*, 143, *167*, 205, *257*  
 Bailey, R. E., 275, 308, 329, *332*  
 Baker, R. E., 43, 45, *49*  
 Balderas, L., 143, *167*  
 Baldes, E. J., 346, *348*  
 Balfour, W. E., 119, *134*  
 Barac, G., 377, *382*  
 Barger, G., 352, *384*  
 Barker, S. B., 353, 356, 357, 358, 359, 363, 378, 379, 380, 381, *382*, *383*, *384*  
 Barlow, J., 304, 305, *331*  
 Barnett, J., 88, *89*, 107, *134*  
 Barnum, C. P., 202, *260*  
 Bartczak, E., 64, 67, *89*  
 Bartter, F. C., 147, *167*, 269, 271, 273, 275, 279, 289, 305, 309, 316, 323, 327, 329, 330, *331*, *332*, *334*  
 Bassde, H. H., 35, *60*  
 Basu, G. K., 240, 241, 243, 244, *263*  
 Bates, R. W., 7, 20, *47*, *48*  
 Bauld, W. S., 3, 9, 15, 16, 17, 19, 20, 35, 36, 37, 40, 46, *47*, *48*, *49*  
 Baulieu, E. E., 192, *197*, 287, 294, 306, 309, 312, *331*  
 Baumann, E., 351, *383*  
 Bayliss, R. I. S., 205, 206, *234*, *257*  
 Beale, D., 364, 380, *383*, *385*  
 Beall, D., 208, *260*  
 Beck, J. C., 189, *196*, 272, *336*  
 Beck, S., 62, *89*  
 Beer, C. T., 6, 12, 13, 25, 32, 40, *48*  
 Bergstrand, C. G., 180, 186, 188, *196*  
 Berkson, J., 346, *348*  
 Berman, R. A., 71, 72, *90*, 148, *168*, 228, *263*  
 Bernstein, D. E., 249, *264*  
 Bertrand, J., 6, 11, 30, 31, 32, 40, 43, 46, *49*, 203, 204, 254, *261*  
 Besli, P. K., 248, 250, *260*  
 Beyth, L., 62, *89*  
 Billeter, J. R., 291, *335*  
 Bird, R., 364, 372, *383*  
 Birke, G., 36, *48*, 180, 186, 188, *196*, 203, 204, 240, 241, 243, 244, 245, *257*, *258*, *262*  
 Birmingham, M. K., 220, 223, *259*  
 Bittner, J. J., 202, *260*  
 Black, E., 254, 255, *258*  
 Blair, H. A. F., 5, 6, 7, *48*  
 Blaschko, H., 345, *347*  
 Blau, N. F., 359, 368, *383*

- Bliss, E. L., 202, 214, 257, 261  
 Bloch, E., 271, 274, 335  
 Block, R. J., 360, 362, 364, 369, 371, 373, 374, 383, 384, 385  
 Bloom, B. R., 193, 197  
 Bloor, W. R., 343, 347  
 Boëtius, M., 374, 383  
 Boettiger, E. G., 44, 48  
 Bojesen, E., 282, 283, 311, 324, 325, 330, 331  
 Bollman, J. L., 345, 346, 348, 353, 383  
 Bondy, P. K., 111, 134, 234, 252, 257, 278, 299, 331  
 Bone, A. D., 341, 344, 345, 346, 347, 348, 349  
 Bongiovanni, A. M., 12, 13, 37, 48, 144, 146, 150, 151, 158, 159, 160, 167, 180, 182, 188, 189, 196, 206, 211, 221, 230, 238, 257, 259, 289, 293, 332  
 Borrell, S., 204, 257  
 Borrowes, E. T., 380, 383  
 Borth, R., 4, 48, 118, 134, 149, 151, 167, 226, 243, 257  
 Bowden, C. H., 370, 374, 383  
 Bowman, R. L., 338, 348  
 Boyle, A. J., 359, 385  
 Braasch, J. W., 361, 383  
 Bradlow, H. L., 193, 194, 197, 200, 203, 205, 212, 259, 260, 303, 332  
 Braunsberg, H., 234, 247, 257  
 Brem, J., 271, 274, 335  
 Breuer, H., 11, 35, 46, 48  
 Britt, R. D., Jr., 331, 333  
 Brodie, B. B., 254, 262, 338, 348  
 Brodie, O. J., 346, 348  
 Brooks, C. J. W., 240, 257  
 Brooks, R. V., 191, 192, 193, 196, 278, 279, 292, 294, 299, 305, 307, 309, 310, 313, 331  
 Brooksbank, B. W. L., 59, 60, 89  
 Brown, C. H., 37, 41, 48, 60  
 Brown, H., 203, 254, 257, 259, 262  
 Brown, J. B., 5, 6, 7, 8, 9, 12, 13, 15, 19, 24, 25, 27, 35, 36, 37, 40, 41, 48, 49, 149, 156, 159, 168  
 Browne, J. S. L., 31, 50, 147, 148, 168  
 Buchborn, E., 304, 307, 332  
 Bucher, N. L., 153, 167  
 Buck, R. R., 219, 261, 276, 334  
 Budnitz, E., 271, 274, 335  
 Buchler, H. J., 205, 206, 257  
 Bulbrook, R. D., 5, 9, 19, 35, 36, 48, 49, 300, 301, 304, 305, 316, 317, 320, 322, 323, 333  
 Bullen, S. S., 343, 347  
 Bunim, J. J., 254, 262  
 Burn, G. P., 341, 347  
 Burstein, S., 6, 31, 48, 53, 54, 59, 80, 89, 204, 207, 208, 211, 229, 257, 258, 294, 332  
 Burton, R. B., 75, 89, 96, 104, 107, 134, 136, 173, 197, 219, 220, 222, 228, 260, 264, 266, 289, 293, 332, 336  
 Busch, R. D., 204, 209, 213, 230, 232, 235, 239, 249, 250, 253, 263  
 Bush, I. E., 32, 33, 48, 96, 98, 103, 116, 134, 188, 196, 200, 209, 211, 214, 225, 236, 243, 249, 258, 266, 274, 277, 278, 280, 287, 289, 292, 293, 295, 298, 303, 308, 309, 310, 311, 329, 332, 335  
 Butenandt, A., 2, 48, 92, 134, 142, 147, 167  
 Butler, A. M., 85, 90  
 Butler, G. C., 150, 167, 227, 251, 258  
 Butler, H. C., 95, 97, 103, 119, 121, 135  
 Butler, P., 271, 274, 335  
 Butt, W. R., 72, 88, 89, 93, 94, 96, 97, 102, 107, 108, 119, 120, 134, 208, 243, 244, 258  
 Ryers, C., 203, 206, 269

## C

- Cahen, R. L., 85, 89  
 Cahill, G., Jr., 275, 332  
 Callow, N. H., 69, 72, 84, 89  
 Callow, R. K., 69, 72, 84, 89, 201, 258  
 Campbell, E., 80, 90  
 Cannon, R. K., 282, 333  
 Cara, J., 144, 146, 151, 167  
 Carballreira, A., 272, 336  
 Carlo, J., 104, 135, 202, 262  
 Carter, A. C., 327, 335  
 Carter, P., 3, 7, 8, 9, 15, 20, 48, 49, 70, 90, 226, 259  
 Cartland, G. F., 266, 332  
 Casida, L. E., 95, 96, 100, 101, 112, 119, 120, 135  
 Casper, A. G. T., 271, 273, 275, 305, 334  
 Cassidy, C. E., 357, 359, 382, 383  
 Castle, H., 202, 261  
 Cates, J. E., 274, 304, 305, 331  
 Cella, J. A., 275, 333  
 Cerreo, E., 93, 94, 98, 99, 100, 101, 108, 109, 117, 135, 143, 168

- Chaikoff, I. L., 352, 363, 368, 376, 384, 385  
 Chaney, A. L., 149, 167, 374, 383  
 Chang, E., 234, 258  
 Chart, J. J., 271, 301, 333  
 Chatin, A., 351, 383  
 Chen, C., 218, 219, 223, 226, 258, 263  
 Chen, P. S., 281, 282, 289, 332  
 Christo, E., 72, 90  
 Christy, N. P., 235, 263  
 Clausen, J., 356, 383  
 Clayton, G. W., 151, 167, 206, 249, 250, 254, 257, 260  
 Cobler, H., 92, 134  
 Code, C. F., 272, 274, 334  
 Coghlan, J. P., 275, 332  
 Cohen, G., 344, 345, 346, 347, 347  
 Cohen, H., 7, 20, 47, 48  
 Cohen, S. L., 6, 30, 31, 48, 149, 167, 204, 205, 207, 209, 211, 258  
 Coindet, J. R., 351, 383  
 Comline, R. S., 119, 134  
 Condon, G. P., 6, 7, 11, 12, 13, 14, 19, 31, 33, 48, 49  
 Conn, J. W., 203, 258, 270, 327, 332, 335  
 Conn, W. M., 149, 168  
 Constandse, M., 214, 262  
 Cook, L. R., 144, 164, 168  
 Cope, C. L., 254, 255, 258, 277, 332  
 Coppage, W. S., 275, 277, 303, 314, 315, 332, 334  
 Corcoran, A. C., 203, 206, 208, 225, 258, 259, 261  
 Corey, K. R., 381, 383  
 Corner, G. W., 92, 134  
 Cornfield, J., 269, 271, 273, 275, 305, 334  
 Coulomb, B., 249, 261  
 Cox, R. I., 150, 151, 161, 167, 172, 173, 174, 179, 180, 183, 186, 188, 189, 193, 193, 196, 197, 200, 204, 205, 226, 227, 229, 251, 258, 259  
 Crabbé, J., 233, 236, 237, 238, 260, 306, 333  
 Craig, L. C., 15, 50, 100, 135  
 Crane, K. F., 102, 109, 110, 117, 136  
 Crépy, O., 31, 34, 48  
 Crout, J. R., 344, 345, 348  
 Dancis, J., 6, 7, 11, 12, 13, 14, 19, 31, 33, 48, 49  
 Dao, T. L., 19, 48  
 Das Gupta, D., 275, 308, 329, 332  
 Daughaday, W. H., 209, 225, 258, 327, 332  
 Davies, P. L., 211, 260  
 Davies, R. E., 11, 19, 43, 49  
 Davis, J. O., 311, 324, 329, 330, 332  
 Davis, M. E., 145, 146, 167  
 Davis, S. A., 275, 332  
 Deanesly, R., 142, 167  
 Decker, H. A., 235, 254, 261  
 De Courey, C. J., 151, 167  
 de Giuseppe, L., 110, 135  
 Degn, H., 282, 311, 324, 330, 331  
 DeGroot, L. J., 368, 383  
 Deiss, W. P., 363, 381, 383, 385  
 DeLaat, B. M., 72, 89  
 Del Greco, F., 203, 258  
 Demetriou, J. A., 12, 13, 49  
 Deming, Q. B., 269, 273, 301, 303, 320, 332, 333  
 Dent, C. E., 380, 383  
 Denton, D. A., 275, 329, 332  
 DeRenzo, E. C., 249, 261  
 Derouaux, G., 339, 348  
 Desaulles, P., 270, 271, 274, 275, 332  
 Deshpande, G. N., 95, 97, 98, 101, 110, 117, 119, 121, 136, 144, 168  
 de Valk, A. T., 346, 348  
 De Vigan, M., 287, 294, 306, 309, 312, 331  
 Davis, R., 72, 89  
 de Walteville, H., 149, 167  
 Dickson, W. M., 95, 97, 103, 119, 121, 135  
 Diezfulusy, E., 15, 19, 27, 29, 30, 31, 32, 33, 35, 36, 41, 43, 44, 47, 48, 49, 93, 98, 99, 100, 101, 112, 113, 114, 135, 180, 196, 204, 234, 240, 241, 243, 244, 245, 257, 258, 262  
 Diengott, D., 183, 197  
 DiMauro, S., 225, 226, 260  
 Dimitriadou, A., 364, 383  
 Dingemans, E., 72, 89  
 Dingman, J. F., 278, 299, 335  
 Di Perri, T., 299, 332  
 Dirks, H. B., Jr., 358, 383  
 Dobriner, K., 53, 89, 204, 205, 227, 242, 259, 260, 261  
 Dodgson, K. S., 79, 89  
 Doe, R. P., 202, 258  
 Doisy, E. A., 2, 48, 49, 205, 206, 257

## D

- Dailey, W., Jr., 275, 308, 329, 332  
 Damast, B., 9, 12, 15, 26, 27, 30, 32, 40, 20

- Doisy, P. P., 205, 257  
 Dominguez, O. V., 203, 262  
 Dorfman, R. I., 53, 59, 60, 61, 62, 63, 72, 73, 74, 75, 89, 90, 142, 143, 167, 191, 193, 194, 196, 197, 201, 204, 208, 210, 211, 214, 229, 242, 249, 250, 253, 254, 257, 258, 260, 261, 262, 267, 268, 270, 332  
 Dowles, A. P., 376, 382  
 Dowling, J. T., 356, 383  
 Doyle, M. L., 205, 260  
 Dragúnová, I., 372, 383  
 Drekter, I., 64, 67, 82, 89  
 Dubravčić, M., 371, 375, 383, 385  
 Duckert-Maulbetsch, A., 233, 236, 237, 238, 260  
 Dufault, F. X., 271, 274, 335  
 Duggan, D. E., 338, 348  
 Dustan, H. P., 206, 259  
 Dyrenfurth, I., 189, 196, 205, 259, 272, 274, 276, 277, 281, 293, 295, 296, 297, 298, 299, 305, 306, 307, 309, 312, 332, 336  
 Dyrenfurth, T., 271, 274, 309, 336
- E**
- Eberlein, W. R., 12, 13, 37, 48, 144, 146, 150, 151, 158, 159, 160, 167, 180, 182, 188, 189, 196, 206, 211, 221, 230, 238, 257, 259, 289, 293, 332  
 Edelhoch, H., 368, 385  
 Edgar, D. G., 94, 96, 97, 103, 104, 119, 120, 135  
 Edwards, R. W. H., 241, 243, 244, 245, 259  
 Ehrlén, I., 338, 339, 344, 348  
 Eichhorn, J., 311, 328, 331  
 Eik-Nes, K., 29, 43, 44, 45, 48, 80, 89, 191, 197, 202, 203, 209, 212, 214, 231, 234, 235, 238, 239, 257, 259, 262  
 Eik-Nes, K. B., 95, 96, 97, 104, 111, 112, 116, 119, 121, 123, 128, 132, 135  
 Eisenberg, J., 170, 197  
 Eitingon, I. V., 204, 224, 227, 263  
 Ekins, R. P., 356, 383  
 Elliott, F. H., 220, 223, 249, 259  
 Ely, R. S., 249, 259  
 Emberland, R., 35, 50  
 Emmens, C. W., 69, 84, 89  
 Engel, L. L., 3, 7, 8, 9, 14, 15, 20, 27, 30, 46, 48, 49, 50, 143, 167, 201, 204, 226, 259, 266, 335  
 Englert, E., Jr., 203, 259  
 Engstrom, L., 44, 47  
 Erb, R. E., 95, 97, 103, 119, 121, 135, 136  
 Ercoli, A., 110, 135  
 Etting, N., 356, 366, 381, 383  
 Eton, B., 37, 41, 47, 119, 128, 136  
 Evans, L. K., 108, 135  
 Evans, W. A., 67, 89  
 Evelyn, K. A., 31, 50, 148, 168
- F**
- Fabrikant, I., 363, 385  
 Fagan, S. S., 203, 258  
 Farran, H. E. A., 364, 372, 383  
 Farrell, G. L., 214, 248, 263, 268, 271, 274, 276, 290, 292, 298, 306, 307, 310, 329, 332, 333, 334  
 Feil, M. L., 267, 268, 270, 332  
 Feldstein, M., 67, 89  
 Fels, E., 92, 135, 136  
 Fernholz, E., 92, 135  
 Fesler, K. W., 278, 299, 335  
 Fevold, H. L., 92, 135  
 Fiebig, E. C., 372, 383  
 Field, E. O., 341, 347  
 Fielding, L. L., 226, 259  
 Fields, M., 224, 259  
 Fieser, L. F., 88, 89, 90, 107, 136, 171, 196, 224, 259  
 Fieser, M., 171, 196  
 Fink, K., 380, 383  
 Fink, R. M., 380, 383  
 Finkelstein, M., 20, 49, 60, 61, 62, 63, 89, 170, 171, 172, 173, 174, 176, 177, 180, 181, 183, 184, 185, 186, 188, 189, 190, 194, 195, 196, 197, 200, 227, 250, 251, 258, 259, 264  
 Fischer, P., 338, 339, 342, 348  
 Fischer, R. H., 149, 167  
 Fischer, R. L., 341, 349  
 Fish, W. R., 142, 167  
 Fishman, J., 3, 6, 15, 24, 25, 27, 35, 49  
 Fishman, W. H., 31, 34, 49  
 Fletcher, K., 372, 383  
 Flink, E. B., 202, 258  
 Flint, M. G., 202, 258  
 Flock, E. V., 346, 348, 353, 361, 383

- Flood, C., 278, 279, 294, 297, 300, 301, 302, 303, 305, 306, 310, 313, 314, 315, 316, 317, 320, 322, 323, 326, 332, 336  
 Florentin, A. A., 202, 261  
 Folch-Pi, J., 209, 259  
 Forbes, T. R., 93, 105, 119, 130, 131, 155, 197  
 Forchielli, E., 59, 60, 61, 62, 63, 89, 90  
 Ford, D. H., 381, 383  
 Forsham, P. H., 209, 259  
 Foss, O. P., 359, 374, 383  
 Foster, G. L., 359, 380, 384  
 Fotherby, K., 56, 57, 89, 151, 161, 167, 194, 197  
 Fortier, C., 250, 259  
 Fortunato, J., 203, 206, 260  
 Frame, E. G., 87, 89  
 Francis, C. M., 12, 13, 37, 48  
 Francis, F. E., 44, 49  
 Franksson, C., 36, 48  
 Fraser, T. R., 364, 383  
 Freedberg, A. S., 356, 384  
 Freeman, S., 226, 263  
 Freinkel, N., 323, 333, 356, 383  
 Fridenson, A., 2, 49  
 Friend, D. G., 346, 349  
 Friis, T., 356, 383  
 Fukushima, D. K., 170, 184, 186, 188, 193, 194, 197, 200, 203, 204, 205, 212, 227, 242, 259, 260, 303, 332  
 Furujhelm, M., 150, 167  
 Fyfe, A., 351, 383
- G**
- Gabrilove, J. L., 170, 197  
 Gaddum, J. H., 338, 348  
 Gale, M., 33, 48  
 Gallagher, T. F., 3, 6, 12, 13, 15, 24, 25, 27, 32, 35, 40, 48, 49, 57, 90, 170, 184, 186, 188, 193, 194, 197, 200, 203, 204, 205, 212, 227, 242, 259, 260, 303, 332  
 Galton, V. A., 361, 362, 365, 383  
 Gann, D. S., 279, 309, 329, 330, 331  
 Gannon, A., 193  
 Ganong, W. F., 275, 308, 329, 332  
 Gardner, L. I., 83, 89  
 Garlick, W. R., 358, 383  
 Garrod, O., 205, 252, 254, 255, 257, 273, 275, 297, 302, 303, 304, 305, 311, 322, 331, 332  
 Garst, J. B., 276, 292, 298, 306, 307, 310, 333  
 Gaunt, R., 271, 275, 333  
 Gawienowski, A. M., 372, 383  
 Geissler, G., 35, 48  
 Gemmill, C. L., 379, 383  
 Genzell, C. A., 202, 234, 259  
 Genest, J., 273, 276, 277, 278, 280, 289, 292, 297, 299, 304, 307, 308, 309, 312, 333, 334  
 Geschickter, C. F., 153, 167  
 Gibson, J. G., 67, 89  
 Gibson, J. K., 241, 242, 243, 244, 257  
 Gibson, R. B., 69, 90  
 Gillam, A. E., 108, 136  
 Ginsburg, N., 92, 93, 94, 96, 106, 136  
 Ginsel, L. A., 378, 384  
 Girard, A., 2, 49, 85, 89, 99, 135, 227, 260  
 Giroud, C. J. P., 271, 272, 274, 275, 308, 309, 328, 329, 332, 333, 335, 336  
 Givner, M. L., 20, 36, 46, 48  
 Glenn, E. M., 214, 234, 235, 260, 263  
 Glick, J. H., 205, 260  
 Gmelin, R., 372, 384  
 Goding, J. R., 275, 332  
 Gold, E. M., 243, 244, 260  
 Gold, J. J., 193, 197  
 Goldberg, S., 172, 177, 180, 181, 194, 197, 251, 259  
 Goldenberg, M., 344, 345, 346, 347, 347  
 Goldner, M., 277, 299, 309, 334  
 Goldzieher, J. W., 20, 43, 45, 46, 48, 49, 248, 250, 260  
 Golodetz, A., 356, 384  
 Golub, O. J., 206, 240, 241, 243, 244, 260, 263, 279, 292, 306, 307, 309, 310, 313, 335  
 Golumbic, C., 100, 135  
 Gordan, E. A., 301, 333  
 Gordon, A. H., 355, 384  
 Gordon, E. E., 22, 49  
 Gordon, S. M., 109, 135  
 Gornall, A. G., 212, 256, 260, 276, 280, 281, 309, 333  
 Gorski, J., 95, 97, 103, 119, 121, 135, 136  
 Gough, N., 149, 152, 153, 168  
 Goulden, F., 72, 90  
 Gowenlock, A. H., 278, 279, 295, 305, 311, 333

- Grant, G. A., 208, 260  
 Grauer, R. C., 80, 90  
 Gray, C. H., 312, 333  
 Greaves, M. S., 316, 333  
 Green, O. C., 189, 197  
 Green, S., 34, 49  
 Greenberg, W., 184, 186, 197  
 Greenblatt, R. B., 191, 193, 197  
 Greenway, R. M., 15, 17, 48  
 Greenwood, F. C., 5, 9, 19, 35, 36, 37, 48, 49, 300, 301, 304, 305, 316, 317, 320, 322, 323, 333  
 Grindley, J. H., 353, 383  
 Grollman, A., 266, 333  
 Gross, J., 353, 355, 361, 363, 364, 376, 381, 383, 384  
 Grossmann, A., 375, 384  
 Grossmann, G. F., 375, 384  
 Grunbach, M. M., 206, 357  
 Grundy, H. M., 274, 333  
 Gsell, M., 149, 167  
 Guerra, S. L., 208, 235, 254, 262, 293, 335  
 Guillemin, R., 249, 250, 254, 260  
 Gut, M., 313, 333  
 Gutbier, G., 374, 383  
 Guterman, H. S., 149, 167  
 Gutiérrez Ríos, E., 371, 373, 384  
 Gwilliam, C., 276, 280, 281, 333
- ## II
- Hackman, R. H., 341, 348  
 Hagedorn, D., 301, 333  
 Hagen, P., 345, 347  
 Hagopian, M., 6, 12, 13, 29, 49, 104, 135  
 Haines, W. J., 298, 333  
 Halberg, F., 202, 210, 250, 260  
 Hale, M. G., 341, 348  
 Halla, M., 15, 27, 49  
 Ham, C. L., 20, 60  
 Hamburg, J., 342, 348  
 Hamburger, C., 65, 89, 245, 260  
 Hamlin, J. T., III, 346, 349  
 Hamolsky, M. W., 356, 384  
 Hamwi, G. J., 186, 197  
 Hanks, L. U., 359, 374, 383  
 Harington, C. R., 352, 368, 384  
 Hariton, L. B., 204, 261  
 Harkness, E. V., 31, 60  
 Harley-Mason, J., 338, 339, 348  
 Harrop, G. A., 266, 333  
 Hartman, F. A., 266, 333  
 Hartmann, M., 92, 135, 141, 167  
 Harwood, C. T., 235, 260  
 Haskins, A. L., 93, 94, 97, 98, 99, 103, 119, 120, 135  
 Haslewood, G. A. D., 59, 60, 89  
 Hayano, M., 194, 197  
 Haydar, N. A., 202, 206, 209, 233, 262  
 Haynes, R., 211, 260  
 Hayward, S. J., 83, 88  
 Heard, R. D. H., 204, 207, 218, 260  
 Hechter, O., 142, 143, 148, 167, 194, 197, 208, 210, 214, 260, 311, 328, 331  
 Heftmann, E., 103, 135  
 Heidt, L. J., 378, 384  
 Heijdemann, S. F. B., 357, 384  
 Heisler, A., 64, 67, 82, 89  
 Hellman, L., 193, 194, 197, 203, 205, 259, 260, 303, 332  
 Hellmann, K., 312, 333  
 Hellström, J., 36, 48  
 Hems, B. A., 380, 383  
 Henly, A. A., 88, 89, 107, 134, 218, 260  
 Hernandez, L., 306, 333  
 Henry, J. S., 147, 168  
 Henry, R. J., 206, 240, 241, 243, 244, 260, 263, 279, 292, 306, 307, 309, 310, 313, 335  
 Herrmann, E., 92, 135  
 Herrmann, W., 151, 167  
 Hershsberg, E. B., 88, 89, 90, 107, 136  
 Hertoghe, J., 233, 236, 237, 238, 260  
 Hesse, R. H., 302, 335  
 Hestrin, S., 61, 89  
 Heusler, K., 291, 335  
 Hill, S. R., Jr., 353, 383  
 Hillen, E., 234, 238, 263  
 Hinsberg, K., 95, 97, 102, 103, 110, 117, 119, 120, 135  
 Hirano, S., 98, 135  
 Hird, F. J. R., 353, 363, 384  
 Hirschmann, F. B., 242, 260  
 Hirschmann, H., 242, 260, 271, 274, 290, 310, 329, 332  
 Hisaw, F. L., 92, 135  
 Hoffmann, H., 277, 333  
 Hohlweg, W., 92, 134, 142, 167  
 Holland, B., 344, 347, 347  
 Hollander, V. P., 225, 226, 260

Holloszy, J., 327, 332  
 Holtorff, A. F., 84, 89  
 Hooker, C. W., 93, 135  
 Horning, E. C., 299, 336  
 Horwitt, B. N., 142, 167  
 Howard, F. A., 346, 347  
 Hsu, C. T., 220, 221, 263, 277, 335  
 Huber, D., 149, 167  
 Hübener, H. J., 117, 135  
 Hughes, E. R., 249, 259  
 Huis in't Veld, L. G., 72, 89, 232, 234, 238, 260, 263  
 Hultberg, S., 36, 38  
 Humphrey, M. J., 358, 383  
 Humphries, P., 204, 261  
 Hunt, C. A., 225, 262  
 Hurlock, B., 21, 49  
 Hurter, R., 304, 333  
 Hutchinson, R., 351, 387

## I

Iannaccone, A., 170, 197  
 Ingall, S., 243, 258  
 Ingbar, S. H., 323, 333, 355, 356, 361, 383, 387  
 Island, D., 277, 303, 314, 315, 332, 337  
 Ittrich, G., 19, 20, 36, 49  
 Izzo, A. J., 219, 220, 221, 222, 260

## J

Jacobs, M., 346, 348  
 Jacobs, S. L., 240, 241, 243, 244, 263  
 Jacobsen, R. P., 210, 214, 260  
 Jacobsohn, G. M., 258  
 Jaffe, H., 225, 258  
 Jailer, J. W., 20, 49, 193, 197, 235, 263  
 James, A. T., 352, 385  
 Janieson, G. S., 352, 385  
 Januszewicz, V., 301, 303, 320, 322, 333  
 Jayle, M. F., 31, 34, 48, 192, 197, 287, 291, 331  
 Jeanloz, R. W., 210, 214, 260  
 Jeffries, J. P., 376, 382  
 Jenkins, D., 202, 209, 232, 233, 262  
 Jensen, C. C., 150, 167, 221, 260  
 Johnson, B. B., 269, 270, 271, 273, 274, 301, 305, 308, 331, 333

Johnson, T. B., 352, 384  
 Johnston, A. G., 380, 385  
 Joly, L.-P., 341, 348  
 Jones, G. E. S., 148, 167  
 Jones, K. M., 300, 301, 304, 305, 316, 317, 320, 322, 323, 333  
 Jones, M. E., 57, 90  
 Jones, R., 276, 278, 280, 289, 312, 334  
 Jones, R. M., 272, 333  
 Jones, R. N., 287, 289, 312, 333, 334  
 Jorgensen, M., 202, 240, 241, 243, 260  
 Junk, R., 117, 135

## K

Kabura, F., 285, 335  
 Kagawa, C. M., 268, 269, 270, 271, 275, 333  
 Kalant, H., 248, 260  
 Kaleita, E., 249, 261  
 Kapphahn, J. L., 21, 49  
 Karrer, A., 208, 235, 262, 293, 335  
 Karaunahatnam, M. C., 206, 260  
 Kassenaar, A., 234, 260  
 Katzman, P. A., 205, 206, 257, 260  
 Kaufmann, C., 98, 119, 135  
 Keis, R. J., 331, 333  
 Kellar, R. J., 149, 165  
 Keller, A. R., 214, 261  
 Keller, G. J., 300, 333  
 Kelley, V. C., 249, 259  
 Kellie, A. E., 72, 79, 89, 241, 243, 244, 245, 259  
 Kelly, W. G., 301, 303, 320, 322, 333  
 Kemp, A. D., 212, 242, 259  
 Kendall, E. C., 352, 370, 376, 384  
 Kennedy, T. H., 362, 384  
 Kcanv, A. P., 72, 89  
 Kent, H. S., 191, 197  
 Kepler, E. J., 205, 227, 251, 261  
 Kernel, L., 243, 244, 258  
 Keston, A. S., 282, 333  
 Keutmann, E. H., 75, 89, 96, 104, 134, 136, 219, 220, 221, 222, 228, 258, 260, 289, 293, 332, 336  
 Kies, M. W., 211, 260  
 Kinsella, R. A., Jr., 44, 49, 205, 257, 260  
 Kirchner, J. G., 300, 333  
 Kirk, M. R., 115, 135

- Klein, D., 109, 135  
 Klein, O., 62, 89  
 Klein, R., 203, 206, 260  
 Klendshoj, N. C., 67, 89  
 Kliman, B., 282, 285, 290, 291, 292, 291,  
     296, 306, 311, 313, 317, 323, 324, 325, 330,  
     332, 333  
 Klitgaard, H. M., 358, 380, 383, 384  
 Kloppe, A., 144, 146, 149, 150, 155, 156,  
     161, 162, 163, 164, 168  
 Kober, S., 147, 167  
 Koch, F. C., 84, 89  
 Koch, W., 61, 89  
 Kochakian, C. D., 116, 135  
 Koczogorek, R., 304, 307, 332  
 Kohler, H., 302, 335  
 Koiv, E., 276, 278, 280, 289, 297, 307, 308,  
     309, 312, 334  
 Kolthoff, I. M., 370, 385  
 Komant, W., 376, 384  
 Kono, T., 360, 364, 372, 373, 384  
 Koritz, S. B., 220, 260, 262  
 Koupreianov, A., 109, 136  
 Kraychy, S., 3, 6, 15, 24, 25, 27, 35, 49  
 Kritchevsky, D., 115, 135  
 Kritchevsky, T. H., 200, 205, 212, 259  
 Kroc, R. L., 380, 385  
 Kruger, F. A., 186, 197  
 Kuizenga, M. H., 266, 332  
 Kulp, J. L., 203, 260  
 Kusch, K., 291, 293, 302, 303, 316, 330  
 Kushinsky, S., 12, 13, 49  
 Kydd, D. M., 360, 384
- L**
- Lafon, M., 352, 370, 376, 384, 385  
 Laidlaw, J. C., 202, 206, 209, 233, 262  
 Lakshmanan, T. K., 72, 78, 80, 89  
 Lambot, H., 339, 348  
 Lampe-Hintzen, D. A. V. M., 232, 234,  
     238, 260, 263  
 Langer, P., 372, 383  
 Lanman, G., 143, 167  
 Laragh, J. H., 291, 301, 303, 304, 305, 314,  
     320, 321, 322, 333, 336  
 Larson, F. C., 381, 383  
 Lassiter, W. E., 365, 384  
 Laumas, K. R., 294, 313, 319, 321, 325, 333,  
     334, 335
- Layne, D. S., 3, 19, 49, 210, 260, 278, 279,  
     294, 297, 300, 301, 302, 303, 304, 305, 310,  
     313, 314, 315, 316, 317, 320, 321, 322, 323,  
     326, 327, 332, 334  
 Leblond, C. P., 352, 363, 384  
 Lecomte, J., 339, 342, 348  
 Leeds, N. S., 200, 212, 259  
 Leftin, J. H., 221, 232, 261  
 Leland, J. P., 359, 380, 384  
 Leon, Y. A., 5, 49  
 Leonard, M. P., 221, 261  
 Leonard, S. L., 92, 135  
 LeRoy, G. V., 285, 335  
 Levitz, M., 6, 7, 11, 12, 13, 14, 19, 25, 26,  
     27, 31, 33, 48, 49  
 Levy, G. A., 206, 260  
 Levy, H., 210, 214, 260  
 Levy, L. K., 6, 12, 13, 14, 29, 49  
 Levy, M., 282, 333  
 Lewbart, M. L., 31, 32, 34, 50, 209, 236,  
     263, 281, 289, 293, 297, 305, 306, 307, 309,  
     312, 316, 334  
 Lewis, C., 21, 49  
 Liddle, G. W., 269, 271, 273, 275, 277, 303,  
     305, 314, 315, 332, 334  
 Lieberman, A. H., 273, 275, 308, 329, 334  
 Lieberman, S., 6, 31, 48, 53, 54, 72, 78, 80,  
     89, 193, 197, 201, 204, 207, 224, 227, 242,  
     258, 259, 260, 261, 291, 301, 302, 303, 304,  
     305, 314, 320, 321, 322, 332, 333, 336  
 Lindberg, M. C., 220, 261  
 Lindeboom, G. A., 357, 384  
 Linder, A., 243, 257  
 Linkvuist, P., 19, 27, 30, 41, 43, 48  
 Lipner, H. J., 359, 379, 380, 382, 383, 384  
 Lipscomb, H. S., 249, 250, 254, 260  
 Lishajko, F., 344, 345, 348  
 Lissitzky, S., 364, 368, 369, 381, 385  
 Litteria, M., 219, 262  
 Little, B., 294, 317, 318, 325, 335  
 Litvak, J., 368, 383  
 Llaurado, J. G., 272, 334  
 Lloyd-Jones, R., 300, 301, 304, 305, 316,  
     317, 320, 322, 323, 333  
 Locher, F., 141, 167  
 Loew, O., 338, 348  
 Loke, K. H., 3, 19, 49  
 Lorraine, J. A., 151, 168  
 Louis, L. H., 203, 258  
 Love, D. N., 194, 197



Lowenstein, B. E., 225, 261  
 Lowry, O. H., 21, 49  
 Loy, R. G., 95, 96, 100, 101, 112, 119, 120, 135  
 Ludwig, W., 369, 384  
 Luetscher, J. A., Jr., 205, 261, 269, 273, 274, 275, 301, 304, 305, 308, 329, 331, 332, 334  
 Lugovoy, J. K., 341, 348  
 Lumry, R., 41, 48, 209, 259  
 Lund, A., 338, 339, 344, 348  
 Lunnon, J. B., 312, 333

## M

McCall, P. J., 57, 90  
 McCollum, S. P., 149, 167  
 MacCorquodale, D. W., 2, 49  
 MacDonald, M. P., 212, 256, 260, 280, 309, 333  
 McGavack, T. H., 64, 67, 82, 89  
 McKee, W. E., 149, 167  
 McKerns, K. W., 249, 261  
 MacLachlan, E. A., 71, 72, 85, 90, 148, 168, 228, 263  
 MacLagan, N. F., 370, 374, 383  
 McLellan, F., 144, 168  
 Macnaughton, M. C., 144, 168  
 McPadden, A. J., 180, 182, 188, 189, 190  
 McShan, W. H., 95, 96, 100, 101, 112, 119, 120, 135  
 Mader, W. J., 219, 261, 276, 334  
 Magee, R. J., 375, 384  
 Magnusson, A. M., 29, 31, 35, 41, 43, 44, 48  
 Mahoney, J. P., 202, 261  
 Man, E. B., 359, 360, 384, 385  
 Mandl, R. H., 360, 362, 369, 371, 373, 374, 383, 384  
 Mangan, G. F., Jr., 345, 346, 347, 348  
 Manger, W. M., 345, 346, 348  
 Mann, W., 352, 384  
 Manning, E. L., 301, 307, 323, 334  
 Marcus, P., 268, 271, 334  
 Marcus, P. I., 21, 50  
 Marenzi, A. D., 378, 384  
 Margraf, H. W., 110, 136, 206, 219, 263  
 Mariz, J. K., 327, 332

Marks, L. J., 221, 232, 261  
 Marrian, G. F., 2, 3, 6, 15, 19, 24, 25, 27, 30, 35, 48, 49, 147, 149, 150, 152, 167, 168, 172, 196, 204, 205, 210, 226, 251, 258, 260, 261  
 Marrian, G. J., 227, 251, 258  
 Marshall, C. W., 210, 214, 260  
 Martin, H., 18, 50  
 Mason, H. L., 205, 206, 210, 226, 227, 242, 251, 259, 261, 272, 274, 290, 296, 334  
 Mason, J. K., 26, 40, 50  
 Mason, J. W., 235, 260, 345, 346, 347, 348  
 Masson, G. M. C., 203, 258  
 Masuda, M., 85, 90  
 Mattingly, D., 191, 192, 193, 196  
 Mattox, V. R., 272, 274, 281, 289, 290, 293, 296, 297, 305, 306, 307, 309, 312, 316, 334  
 Maughan, G. B., 148, 168  
 Mead, R. C., 357, 384  
 Meister, P., 98, 99, 101, 135  
 Melby, J. C., 281, 316, 334  
 Mellinger, R. C., 233, 263  
 Mendel, L. B., 352, 385  
 Menini, E., 31, 32, 33, 35, 44, 48, 49  
 Meslin, F., 31, 34, 48  
 Meyer, A. S., 13, 19, 26, 49, 208, 211, 220, 261  
 Meyer, C., 327, 334  
 Meyer, R. K., 269, 271, 333  
 Michel, R., 352, 353, 357, 364, 365, 368, 369, 370, 376, 381, 384, 385  
 Michie, E. A., 146, 149, 156, 159, 168  
 Miescher, P., 60, 90  
 Migeon, C. J., 6, 11, 19, 30, 31, 32, 40, 43, 46, 49, 50, 80, 81, 90, 189, 197, 202, 203, 204, 209, 214, 235, 254, 259, 261  
 Mighton, H., 100, 135  
 Millar, R., 346, 348  
 Miller, A. M., 242, 261  
 Miller, J. M., 300, 333  
 Mills, I., 316, 323, 327, 334  
 Mills, I. H., 191, 192, 193, 196  
 Mills, J. H., 279, 309, 329, 330, 331  
 Milne, I. G., 36, 48  
 Mitchell, F. L., 11, 19, 35, 43, 48, 49  
 Monclon, F., 210, 249, 250, 253, 254, 261, 262  
 Mond, B., 242, 261  
 Money, W. L., 6, 7, 11, 19, 31, 33, 48, 49  
 Montagu, K. A., 345, 348

Moolenaar, A. J., 234, 260, 280, 289, 292,  
306, 309, 334  
Morávek, V., 256, 261  
Morreale de Escobar, G., 371, 373, 384  
Morren, H., 377, 382  
Morris, C. J. O. R., 72, 88, 89, 93, 94, 96,  
97, 102, 107, 108, 119, 120, 134, 208, 213,  
256, 258, 261  
Morris, P., 93, 94, 96, 97, 102, 107, 108,  
119, 120, 134  
Morris, R., 208, 243, 244, 258  
Morton, M. E., 352, 376, 384  
Moser, H., 377, 384  
Moxham, A., 244, 261  
Mueller, G. C., 44, 50  
Muller, A. F., 233, 236, 237, 238, 260, 301,  
307, 323, 334  
Mulrow, P. J., 275, 308, 329, 332, 334  
Munch, J. C., 341, 348  
Munkner, T., 356, 383  
Munson, P. L., 57, 90  
Murawec, T., 111, 134, 136  
Murray, G. R., 352, 384  
Myers, G. B., 359, 385

## N

Nabarro, J. D. N., 244, 261, 304, 333  
Nadeau, G., 341, 348  
Nadel, E. I., 204, 208, 258  
Nasutavicus, W., 12, 13, 49  
Natelson, S., 341, 348  
Nathanson, I. T., 7, 8, 9, 15, 20, 48  
Neher, G. M., 98, 99, 101, 102, 135  
Neher, R., 103, 105, 106, 115, 116, 119, 130,  
131, 135, 136, 137, 205, 210, 261, 276, 278,  
280, 282, 287, 289, 290, 291, 293, 295, 297,  
298, 299, 306, 307, 308, 309, 310, 312, 327,  
328, 329, 334, 335, 336  
Nelson, D. H., 80, 89, 90, 116, 136, 202,  
203, 208, 210, 214, 234, 235, 238, 257, 259,  
260, 261, 262, 263, 306, 333  
Nesemann, G., 117, 135  
Newman, A. G., 329, 334  
Nichell, G. J., 206, 259  
Nicholson, W. M., 266, 333  
Nissen-Meyer, R., 243, 262  
Noall, M. W., 93, 98, 99, 101, 102, 135, 136  
Nocke, L., 11, 46, 48

Nocke, W., 35, 48  
Norymberski, J. K., 211, 240, 241, 242, 243,  
244, 257, 258, 261, 262  
Notcher, V., 189, 196  
Nowaczynski, W. J., 276, 277, 278, 280,  
287, 289, 297, 299, 307, 308, 309, 312, 334,  
335  
Nubel, R. C., 116, 136  
Nugent, C. A., 191, 197  
Numeroff, M., 232, 262  
Nunez, J., 353, 364, 365, 385  
Nyland, J., 234, 260  
Nyman, M. A., 43, 45, 49

## O

O'Connor, D., 355, 384  
Odell, A. D., 30, 48, 147, 168  
O'Donnel, J., 118, 136  
Oertel, G. W., 29, 43, 45, 49, 95, 96, 97,  
104, 111, 112, 116, 119, 121, 123, 128, 129,  
132, 135  
Ogata, A., 98, 135  
Okita, J. J., 285, 335  
Olin, J. S., 344, 348  
Oncley, J. L., 30, 50  
Oneson, I. B., 6, 31, 48, 207, 209, 258  
Ong, S., 85, 90  
Orvis, A. L., 272, 335  
Osborn, S. B., 247, 257  
Oslapas, R., 204, 209, 230, 249, 250, 253,  
263  
Osterberg, A. E., 376, 384  
O'Sullivan, D. G., 115, 135, 234, 262  
Oswald, A., 351, 384  
Ott, D., 110, 135  
Ozaki, T., 342, 348

## P

Page, I. H., 208, 225, 258, 261  
Page, J. E., 380, 383  
Paget, M., 338, 348  
Panattoni, M., 19, 49  
Papadatos, C., 203, 206, 260  
Parent, C., 200, 203, 212, 225, 228, 262  
Paterson, J. Y. F., 205, 226, 262  
Patti, A. A., 233, 263  
Paul, A. C., 235, 254, 261

Pearlman, W. H., 67, 86, 90, 93, 94, 97, 98, 99, 100, 101, 108, 109, 117, 135, 143, 168, 205, 254, 255, 257, 302, 303, 322, 331  
 Pearson, O. H., 9, 12, 15, 26, 27, 30, 32, 40, 50, 184, 186, 197, 225, 226, 260  
 Pearson, S., 64, 67, 82, 89  
 Pechet, M. M., 302, 335  
 Pelzer, H., 95, 97, 102, 103, 110, 117, 119, 120, 135  
 Perlmutter, M., 232, 262  
 Péron, F. G., 204, 208, 210, 211, 214, 220, 233, 249, 250, 253, 254, 260, 261, 262, 290, 335  
 Petermann, M. L., 355, 385  
 Peters, J. P., 204, 262, 360, 384  
 Peterson, R. E., 65, 66, 85, 90, 202, 208, 210, 235, 236, 248, 249, 250, 254, 255, 260, 262, 282, 285, 290, 291, 292, 293, 294, 296, 301, 303, 306, 311, 313, 317, 320, 323, 324, 325, 329, 330, 332, 333, 335  
 Pfeiffer, V., 62, 89  
 Pierce, C. E., 65, 66, 85, 90  
 Pincus, G., 67, 72, 73, 74, 75, 86, 90, 104, 135, 142, 167, 200, 202, 208, 210, 211, 212, 214, 225, 226, 228, 260, 262, 268, 271, 303, 327, 334, 335  
 Pincus, J. B., 341, 348  
 Pinto, A., 83, 88  
 Pittman, C. S., 353, 383  
 Pittman, J. A., Jr., 353, 383  
 Pitt-Rivers, R., 352, 353, 355, 361, 362, 364, 365, 370, 376, 383, 384, 385  
 Plager, J. E., 80, 81, 90, 202, 225, 226, 262  
 Plantin, L. O., 36, 48, 180, 186, 188, 196, 203, 204, 240, 241, 243, 244, 245, 257, 258, 262  
 Plotz, E. J., 145, 146, 167  
 Pond, M. H., 72, 90  
 Pont, M. E., 270, 335  
 Porter, C. C., 212, 215, 231, 232, 235, 236, 239, 256, 262, 263, 287, 335  
 Postel, S., 368, 383  
 Potter, V. R., 358, 385  
 Potts, A. M., 267, 268, 270, 332  
 Preedy, J. R. K., 5, 6, 7, 9, 16, 20, 23, 24, 26, 28, 29, 33, 34, 35, 37, 38, 39, 40, 41, 42, 45, 47, 48, 49, 50  
 Prelog, V., 98, 99, 101, 135  
 Price, H. L., 345, 346, 348  
 Price, M. L., 345, 346, 348

Prunty, F. I. G., 191, 192, 193, 196  
 Prystowsky, H., 203, 204, 254, 261  
 Pugh, C. E., 341, 348  
 Puntereri, A., 203, 206, 260  
 Purdy, R. H., 30, 50  
 Purves, H. D., 362, 384

## R

Raab, W., 343, 348  
 Raben, M. S., 357, 359, 382  
 Radichevich, I., 373, 383  
 Raeside, J. I., 94, 97, 103, 119, 120, 136  
 Rall, J. E., 355, 356, 365, 368, 382, 385  
 Ramcharan, S., 278, 279, 294, 297, 300, 301, 302, 303, 305, 310, 313, 314, 315, 316, 317, 320, 322, 323, 326, 332  
 Randolph, J. D., 149, 168  
 Raper, H. S., 341, 348  
 Rasch, G., 65, 89  
 Rauschkolb, E. W., 271, 274, 290, 310, 329, 332  
 Ravenni, G., 299, 332  
 Rawson, R. W., 365, 385  
 Recknagel, R. O., 219, 262  
 Reddy, W. J., 202, 206, 209, 232, 233, 234, 236, 262, 306, 333  
 Redgate, E. S., 329, 334  
 Reese, M., 359, 385  
 Reich, H., 102, 109, 110, 116, 117, 136, 208, 262  
 Reich, M., 309, 329, 335  
 Reichstein, T., 101, 116, 136, 170, 171, 172, 197, 200, 227, 251, 259, 266, 276, 280, 282, 287, 290, 281, 293, 335, 336  
 Reineke, E. P., 369, 378, 385  
 Reineke, L. M., 103, 116, 136  
 Reinwein, H., 118, 136  
 Reith, H., 374, 383  
 Arnold, A. E., 202, 209, 233, 262, 306, 333  
 Rentler, F. W., 346, 349  
 Renzl, A. A., 271, 333  
 Reynolds, S. R. M., 92, 93, 94, 96, 100, 136  
 Rhoads, C. P., 204, 261  
 Richards, J. B., 268, 332  
 Richardson, A. K., 346, 348  
 Richardson, J. A., 346, 348  
 Riegel, I. L., 44, 50  
 Rigby, B., 301, 335  
 Riggs, D. S., 359, 385

- Rinsler, M. G., 301, 335  
 Riondel, A. M., 243, 257, 300, 301, 304,  
 305, 307, 316, 317, 320, 322, 323, 333, 334  
 Robbe, H., 180, 196  
 Robbie, W. A., 69, 90  
 Robbins, J., 355, 368, 372, 385  
 Roberts, N. R., 21, 49  
 Roberts, S., 201, 209, 262, 263  
 Robinson, A. M., 72, 89, 90  
 Roche, J., 352, 353, 357, 364, 365, 368, 369,  
 376, 381, 385  
 Rodgers, J., 144, 168  
 Rodriguez, R. M., 200, 202, 208, 211, 212,  
 225, 228, 262, 303, 335  
 Rogina, B., 375, 385  
 Romanoff, E. B., 104, 135, 225, 262  
 Romanoff, L. P., 200, 202, 208, 210, 211,  
 212, 214, 225, 226, 228, 262, 268, 271, 303,  
 334, 335  
 Rosenberg, E., 271, 274, 304, 321, 335  
 Rosenfeld, G., 289, 332  
 Rosenkrantz, H., 219, 220, 262  
 Ross, E. J., 306, 333  
 Rossclot, J. P., 193, 197  
 Rossipal, E., 278, 279, 291, 297, 300, 301,  
 302, 303, 305, 310, 313, 314, 315, 316, 317,  
 320, 322, 323, 326, 332  
 Roth, G. M., 346, 348  
 Row, V. V., 373, 383  
 Royce, P. C., 271, 274, 290, 310, 329, 332  
 Rubegni, M., 299, 332  
 Rubin, B. L., 72, 73, 74, 75, 90  
 Rudy, M., 279, 292, 306, 307, 309, 310, 313,  
 335  
 Ruggieri, P., 110, 135  
 Ruppert, A., 85, 90  
 Ruschig, H., 92, 136  
 Rutgers, I. J. J., 2, 49  
 Ryan, J., 205, 263  
 Ryan, K. J., 61, 90  
 Salhanick, H. A., 93, 98, 99, 101, 102, 135,  
 136  
 Salter, W. T., 85, 89  
 Saltzman, A. H., 212, 263  
 Salvesen, S., 243, 262  
 Samuels, L. T., 44, 48, 80, 89, 90, 93, 98,  
 101, 123, 136, 191, 197, 202, 203, 208, 209,  
 210, 214, 234, 235, 238, 254, 257, 259, 261,  
 262, 263  
 Sandberg, A. A., 6, 11, 12, 13, 14, 26, 30, 32,  
 40, 43, 44, 45, 50, 202, 209, 214, 235, 254,  
 257, 258, 259, 261, 263, 295, 309, 310, 311,  
 327, 332, 335  
 Sandell, E. B., 370, 385  
 Sandor, T., 289, 312, 334  
 Sandorfy, C., 287, 333  
 Sandulesco, G., 2, 49, 85, 89, 90, 135, 227,  
 260  
 Sanfilippo, S. J., 102, 109, 110, 117, 136  
 Sardinas, J. L., 116, 136  
 Savard, K., 61, 90, 104, 136, 143, 167, 209,  
 211, 260, 263  
 Schally, A. V., 220, 223, 249, 259, 262, 328,  
 335  
 Schedl, H. P., 289, 332  
 Schellman, J. A., 44, 48, 209, 259  
 Schenker, V., 210, 214, 260  
 Schiedt, U., 118, 136  
 Schild, H., 338, 348  
 Schindler, O., 116, 136, 276, 282, 287, 290  
 291, 293, 335  
 Schmidlin, J., 291, 335  
 Schmidt, H., 295, 309, 335  
 Schmidt, J., 92, 134, 147, 167  
 Schmidt-Thomé, J., 117, 135  
 Schneider, J. J., 31, 32, 34, 50, 209, 236,  
 263  
 Schneider, R., 242, 259  
 Schönbaum, E., 220, 223, 259  
 Schöninger, W., 374, 385  
 Schopman, W., 234, 238, 263  
 Schroeder, M. S., 149, 167  
 Schubert, K., 61, 90  
 Schuler, W., 270, 271, 332  
 Schwarz, H., 276, 292, 298, 306, 307, 310,  
 333  
 Seism, G. R., 64, 67, 82, 89  
 Scott, J. F., 20, 50  
 Seelye, J. M., 202, 208, 211, 212, 262, 303,  
 335

## S

- Saba, N., 194, 197, 311, 328, 331  
 Saffan, B. D., 37, 41, 48, 50  
 Saffran, J., 201, 262  
 Saffran, M., 201, 223, 262, 328, 335  
 Sahrholz, F. G., 117, 135  
 Saier, E., 80, 90

- Seki, T., 14, 50  
 Seltze, H. S., 203, 258  
 Serbin, R. A., 186, 197  
 Seuken, A., 95, 97, 102, 103, 110, 117, 119, 120, 135  
 Sha, J., 344, 347, 347  
 Shaw, F. H., 343, 348  
 Shaw, W. H. C., 376, 382, 385  
 Shearman, R. P., 164, 168, 193  
 Sherman, A. I., 93, 103, 135  
 Shipley, E. G., 269, 271, 301, 333  
 Shipley, R. A., 53, 89, 142, 167, 191, 196  
 Shmagranoff, G. L., 308, 329, 334  
 Shoenberger, J., 183, 185, 197  
 Shoppee, C. W., 19, 50, 266, 335  
 Shore, P. A., 344, 348  
 Short, R., 143, 168  
 Short, R. V., 37, 41, 47, 95, 98, 103, 110, 111, 112, 119, 121, 123, 127, 132, 133, 134, 136  
 Shull, G. M., 116, 136  
 Shumway, N. P., 276, 292, 298, 306, 307, 310, 333  
 Sic, H.-G., 31, 49  
 Siegel, H., 372, 383  
 Silber, R. H., 202, 204, 209, 210, 212, 213, 215, 230, 231, 232, 235, 236, 239, 249, 250, 253, 256, 260, 262, 263, 287, 335  
 Silverman, L., 151, 167  
 Simmer, H., 94, 95, 96, 97, 103, 109, 111, 112, 114, 117, 119, 121, 123, 136, 137  
 Simmer, I., 95, 96, 109, 117, 121, 136  
 Simons, E. L., 203, 259  
 Simpson, G. K., 380, 385  
 Simpson, S. A., 116, 134, 136, 211, 252, 254, 255, 257, 263, 268, 270, 271, 272, 273, 274, 276, 278, 279, 280, 282, 283, 287, 290, 291, 293, 297, 301, 308, 311, 314, 329, 331, 333, 335, 336  
 Singer, B., 267, 272, 273, 289, 301, 308, 329, 335, 336  
 Skrubc, H., 359, 385  
 Slade, C. L., 308, 329, 334  
 Slater, J. D. H., 364, 383  
 Slater, S., 232, 262  
 Slaunwhite, W. R., Jr., 6, 7, 8, 9, 11, 12, 13, 14, 15, 20, 26, 30, 32, 40, 43, 44, 45, 48, 50, 234, 258, 327, 335  
 Slotta, K. H., 92, 135, 136  
 Smith, D. C., 204, 240, 263  
 Smith, D. F., 235, 254, 261  
 Smith, J. D., 180, 182, 188, 189, 196, 249, 250, 254, 260  
 Smith, M., 314, 315, 332  
 Smith, W. R., 233, 263  
 Smyles, E., 242, 261  
 Snedecor, G. W., 121, 136  
 Sobel, C., 206, 240, 241, 243, 244, 260, 263, 279, 292, 306, 307, 309, 310, 313, 335  
 Sobel, H., 204, 208, 218, 260  
 Soffer, L. J., 170, 197  
 Soley, M. H., 358, 383  
 Sommerville, I. F., 95, 97, 98, 101, 110, 117, 119, 121, 136, 144, 149, 152, 153, 168  
 Somogyi, M., 237, 263  
 Spencer, A. G., 269, 335  
 Spencer, B., 79, 89  
 Spitzer, J. R., 11, 12, 13, 14, 25, 26, 27, 49  
 Spitzzy, H., 359, 375, 384, 385  
 Spoor, H. J., 266, 333  
 Sprague, A., 67, 89  
 Stachenko, J., 308, 328, 329, 333, 335  
 Stack-Dunne, M. P., 273, 289, 308, 329, 335  
 Stanbury, J. B., 365, 368, 383, 384  
 Stanley, P. G., 372, 383  
 Starr, P., 243, 244, 260  
 Stasilli, N. R., 380, 385  
 Staub, M. C., 278, 299, 335  
 Staudinger, H. J., 277, 295, 309, 333, 335  
 Stein, M., 356, 384  
 Steinbeck, A. W., 234, 257  
 Stempfell, R. S., 203, 204, 254, 261  
 Stern, M. I., 151, 159, 160, 168, 188, 196  
 Stern, S., 64, 67, 82, 89  
 Steyernark, P. R., 276, 278, 280, 287, 289, 298, 312, 334, 335  
 Stidworthy, G., 116, 185  
 Stummel, B. F., 149, 168  
 Stimson, M., 118, 136  
 Stoa, K. F., 35, 50  
 Stokem, M. B., 200, 212, 242, 259  
 Stoken, M., 205, 259  
 Stole, V., 372, 385  
 Stormshak, F., 121, 136  
 Strauss, H., 266, 333  
 Streeten, D. H. P., 270, 335  
 Strickler, H. S., 242, 261  
 Strickler, M. T., 80, 90  
 Strong, J. A., 144, 164, 168

Stubbs, R. D., 240, 241, 242, 243, 244, 257, 261, 262  
 Suzuki, T., 342, 348  
 Sweat, M. L., 203, 214, 236, 248, 249, 263  
 Swyer, G. I. M., 188, 196  
 Stylianou, M., 59, 90  
 Svendsen, R., 283, 285, 335  
 Sybulski, S., 189, 196  
 Szego, C. M., 201, 206, 209, 210, 262, 263

## T

Tait, J. F., 116, 134, 136, 205, 211, 252, 257, 263, 268, 270, 271, 272, 273, 274, 276, 278, 279, 280, 282, 283, 285, 287, 290, 291, 293, 294, 296, 297, 300, 301, 302, 303, 304, 305, 306, 308, 310, 311, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 325, 326, 327, 328, 329, 331, 332, 333, 334, 335, 336  
 Tait, S. A., 205, 254, 255, 257  
 Tait, S. A. S., 278, 279, 280, 294, 296, 297, 300, 301, 302, 303, 304, 305, 306, 310, 311, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 325, 326, 328, 331, 332, 333, 334, 335, 336  
 Talalay, P., 21, 49, 50  
 Talbot, N. B., 71, 72, 85, 90, 109, 136, 148, 168, 204, 205, 212, 224, 227, 228, 263  
 Tata, J. R., 356, 360, 365, 379, 385  
 Taurog, A., 363, 365, 368, 385  
 Taylor, W., 146, 168  
 Teich, S., 201, 261  
 Tewell, H. E., Jr., 218, 219, 223, 258  
 Tewesbury, L. B., 352, 384  
 Thayer, S. A., 2, 48, 49  
 Thomas, M., 97, 100, 135  
 Thorn, G. W., 202, 209, 232, 233, 262, 266, 306, 333  
 Thuline, H. C., 85, 90  
 Tillinger, K. G., 15, 27, 31, 33, 35, 44, 48  
 Titus, E., 100, 135  
 Todd, A. R., 341, 348  
 Tompsett, S. L., 204, 205, 240, 263  
 Tong, W., 368, 385  
 Touchstone, J. C., 111, 133, 136, 220, 221, 263, 277, 335  
 Traill, D., 380, 385  
 Trikojus, V. M., 353, 363, 377, 384, 385  
 Tripod, J., 270, 271, 332  
 Troen, P., 207, 263  
 Trolle, D., 150, 168

Tummillo, M., 59, 90  
 Turner, C. W., 94, 97, 103, 119, 120, 136, 369, 378, 385  
 Turner, P. C. R., 364, 383  
 Twombly, G. H., 11, 12, 13, 14, 25, 26, 27, 49  
 Tyler, F. H., 191, 197, 202, 203, 209, 234, 254, 257, 259, 261, 262, 263

## U

Udenfriend, S., 282, 333, 338, 348  
 Ueberwasser, H., 291, 335  
 Uete, T., 274, 275, 336  
 Ulick, S., 109, 136, 291, 293, 301, 302, 303, 304, 305, 314, 316, 320, 321, 322, 323, 333, 336  
 Umberger, E. J., 110, 117, 136  
 Underwood, R., 294, 300, 301, 306, 336  
 Ungar, F., 193, 197

## V

Vallin, Y., 192, 197  
 Van Arman, C. G., 268, 270, 275, 333  
 Vanden Heuvel, W. J. A., 299, 336  
 van der Vies, J., 234, 238, 263  
 Vande Wiele, R., 193, 197  
 Van Middlesworth, L., 360, 364, 372, 373, 384  
 Van Slyke, D. D., 204, 262, 359, 374, 383  
 Van Wyk, J. J., 206, 257  
 Veler, C. D., 2, 48  
 Vendsalu, A., 346, 347, 348  
 Venning, E. H., 31, 50, 92, 136, 147, 148, 168, 189, 196, 204, 205, 208, 212, 226, 259, 260, 263, 267, 271, 272, 273, 274, 275, 276, 277, 281, 293, 295, 296, 297, 298, 299, 301, 305, 306, 307, 309, 312, 332, 335, 336  
 Verboom, E., 150, 168  
 Verly, W., 343, 348  
 Vermeulen, E., 206, 221, 263  
 Vestergaard, P., 67, 90, 234, 263  
 Vieregiver, E., 148, 167  
 Villalonga, F., 378, 384  
 Villee, C. A., 22, 49  
 Virtanen, A. I., 372, 384  
 Vogt, M., 249, 263  
 von Euler, U. S., 342, 344, 345, 347, 348  
 von Euw, J., 101, 116, 136, 170, 171, 172;

197, 200, 227, 251, 259, 276, 280, 282, 287,  
290, 291, 293, 335, 336  
von Münstermann, A. M., 98, 105, 106,  
109, 113, 114, 117, 119, 123, 130, 131, 137  
von Mutzenbecher, P., 369, 384

## W

Wade, A. P., 72, 79, 89, 243, 244, 259  
Wakim, K. G., 345, 346, 348  
Walker, G., 205, 244, 254, 255, 257, 261,  
302, 303, 304, 305, 322, 331  
Wall, P. E., 6, 11, 19, 30, 31, 32, 40, 43, 46,  
49, 50, 203, 204, 254, 261  
Wallace, E. Z., 235, 263  
Wallach, S., 203, 259  
Wallerstein, J. S., 341, 348  
Warren, F. L., 72, 88, 89  
Warren, S. L., 352, 384  
Watson, E. J. D., 3, 19, 49  
Weichselbaum, T. E., 110, 136, 206, 219,  
263  
Weil-Malherbe, H., 341, 344, 345, 346,  
347, 348, 349  
Weiner, J. S., 312, 333  
Weiner, N., 109, 135  
Weinman, S. H., 192, 197  
Weiss, S. P., 95, 96, 97, 104, 111, 112, 116,  
119, 121, 128, 132, 135  
Welch, A. D., 345, 347  
Wentworth, W. E., 331, 333  
Werbin, H., 85, 90  
Werner, S. C., 360, 364, 369, 373, 383, 385  
West, C. D., 9, 12, 15, 26, 27, 29, 30, 32, 40,  
43, 45, 49, 50  
West, H. F., 240, 262, 316, 333  
Westman, A., 15, 27, 31, 33, 35, 36, 44, 48,  
180, 196, 243, 258  
Westphal, U., 92, 134, 142, 150, 153, 167,  
168  
Wettstein, A., 116, 135, 136, 276, 278, 280,  
282, 287, 289, 290, 291, 293, 295, 297, 298,  
306, 307, 308, 309, 310, 312, 327, 328, 329,  
334, 335, 336  
Wheeler, H. L., 352, 385  
Wheeler, J., 219, 223, 226, 258, 263  
Whitehead, J. K., 282, 283, 314, 331, 364,  
380, 383, 385  
Whitehorn, J. C., 343, 349  
Wieland, P., 291, 335

Wiest, W. G., 94, 104, 105, 107, 117, 136  
Wiggins, R. A., 33, 34, 50  
Wilkins, L., 189, 191, 197  
Wilkinson, J. H., 370, 374, 383  
Willard, H. H., 359, 385  
Willardson, D. G., 203, 234, 254, 257, 259,  
261  
Williams, D. C., 35, 48, 93, 94, 96, 97, 102,  
107, 108, 119, 120, 134, 208, 213, 256, 258,  
261  
Williams, E. S., 285, 335  
Williams, R. H., 225, 258  
Williamson, B., 15, 50  
Willoughby, M. L. N., 188, 196, 200, 243,  
258, 277, 280, 287, 303, 332  
Wilson, H., 70, 90, 225, 226, 263  
Winikoff, D., 377, 385  
Winnick, T., 380, 384  
Wintersteiner, O., 92, 134, 136  
Wurz, H., 266, 268, 270, 336  
Wiswell, J. G., 101, 136  
Wixom, R. L., 212, 263  
Wolf, R. S., 214, 262  
Wolf, W., 353, 364, 385  
Wolfe, J. K., 88, 89, 90, 107, 136, 148, 168,  
205, 212, 263  
Wolff, H. P., 304, 307, 332  
Wolfson, W. Q., 209, 264  
Wood, M., 72, 90  
Woodford, M., 274, 333  
Wotiz, H. H., 18, 50  
Wright, R. D., 275, 333  
Wu, J., 12, 13, 49  
Wynngaarden, J. B., 236, 254, 255, 262  
Wynn, J., 363, 364, 372, 373, 385

## Y

Yankopoulos, N. A., 311, 324, 329, 330, 332  
Yatsu, F. M., 329, 334  
Young, J. G., 341, 349  
Yuen, V. S., 275, 308, 329, 332

## Z

Zachariac, F., 181, 197  
Zaffaroni, A., 75, 89, 96, 104, 107, 111, 116,  
134, 135, 173, 197, 201, 208, 210, 228, 258,  
262, 264, 266, 289, 293, 332, 336

Zak, B., 359, 385

Zander, J., 94, 96, 97, 98, 99, 100, 103, 104,  
105, 106, 109, 111, 112, 114, 117, 119, 120,  
123, 130, 131, 132, 135, 136, 137

Zarrow, M. X., 93, 98, 99, 101, 102, 135,  
136

Zenker, N., 249, 264

Zileli, M. S., 346, 349

Zimmermann, W., 52, 85, 90, 201, 212, 240,  
264

Zondek, B., 170, 172, 177, 181, 183, 197,  
250, 251, 264

Zumoff, B., 303, 332

Zyguntowicz, A. S., 72, 90, 109, 136



# SUBJECT INDEX

## A

Abortion, pregnanediol and, 164

Acetaldehyde,

adrenocorticosteroids and, 215, 217, 226-227, 229-230

pregnanetriol assay and, 151, 161

pregnanetriolone assay and, 172, 176, 179-180

Acetic acid,

aldosterone isolation and, 311, 324

catechol amine assay and, 344, 347

corticosteroids and, 143

estrogen separation and, 33

iodoamino acid separation and, 362, 364-366

urine storage and, 204

Acetic anhydride,

radioactive,

aldosterone and, 282, 290, 302, 311, 314, 323, 330

iodoamino acids and, 380

tetrahydroaldosterone and, 315

Acetone,

adrenocorticosteroid assay and, 209, 210, 232, 239

aldosterone isolation and, 295, 298, 328

estrogen extraction by, 44

pregnanediol isolation and, 148

progesterone extraction and, 97

3 $\alpha$ -Acetoxy-5 $\beta$ -androst-9(11)-en-17-one,

determination of, 76

Acetrizoate, protein and, 357

$\alpha$ -(N-Acetyl)- $\epsilon$ -(N-acetyldiiodotyrosyl)-

lysine, coupling of, 352

Acetylation,

aldosterone and, 274, 276, 290-291, 310-311, 313, 317, 325

pregnanediol isolation and, 150, 157

progesterone identification and, 117, 128, 134

tetrahydroaldosterone isolation and, 315

N-Acetyldiiodotyrosine, coupling of, 352

N-Acetyldiiodotyrosylglutamic acid,

coupling of, 352

Addison's disease, 201

Adrenal cancer,

cortisol and, 184-185

17-ketosteroids and, 63, 183

pregnanetriol and, 182-185, 227

pregnanetriolone and, 171-172, 182-186, 227

Adrenal gland,

adrenocorticosteroids in, 220, 253

aldosterone production by, 275, 308-311, 327-331

estrogens and, 2, 43

17-ketosteroids and, 52

pregnanediol and, 144

pregnanetriol in, 251

steroid extraction from, 209-210

steroidogenesis in, 143-144

Adrenal hyperplasia, 201

adrenocorticosteroids and, 221

enzyme deficiency in, 194

ketosteroids and, 83-84, 187

pregnanetriol and, 146, 151, 164, 182, 187-188

pregnanetriolone and, 170, 171, 186-189, 251

urinary steroids in, 227, 230

virilism and, 144, 151

Adrenaline,

assay,

colorimetric methods of, 341-343

fluorometric methods of, 344-347

chemistry of, 337-338

ethylenediamine conjugation and, 339-341

fluorescence of, 338

Adrenal insufficiency, 201

Adrenochrome, formation of, 338-339, 341

Adrenocorticosteroids, *see also specific compounds*

assay,

chemical methods, 218-247

fluorometric methods, 247-254

general remarks, 199-203, 215-218

other methods, 256

radioisotopic methods, 254-256

- column chromatography of, 213-214
- extraction,
  - hydrolysis and, 204-207
  - purification and, 211-214
  - solvents and, 207-211
- paper chromatography of, 227, 228, 235-236
  - blue tetrazolium and, 221
  - stability of, 204-205
- Adrenocorticotrophic hormone,
  - corticosteroids and, 143, 144
  - corticosterone and, 249
  - Cushing's syndrome and, 189-191
  - 17-ketosteroids and, 181
  - Porter-Silber reaction and, 235
  - pregnanetriolone and, 181, 182, 194
  - virilism and, 192-193, 195
- Adrenogenital syndrome, pregnanetriol in, 147, 150, 182
- Adrenolutin, formation of, 338-339
- Adrenosterone, paper chromatography of, 292
  - separation of, 105
- Albumin, iodoamino acids and, 354-356
- Aldosterone, 170
  - antagonists of, 266
  - assay,
    - adrenal gland, 327-331
    - biological, 266-275, 308-310
    - blood, 275, 323-327
    - interfering compounds and, 312-313
    - physicochemical methods, 276-293
    - purification and, 293-300
    - response parameter and, 272-273
    - urine, 305-318
  - biosynthesis of, 328
  - derivatives of, 290-293, 298, 310-311
  - elution from paper, 298-299
  - fluorescence of, 252, 278-280, 293, 299, 307-311, 313, 317, 328-330
  - glucuronide metabolites of, 314-316
  - identification of, 286-293
  - lactones of, 290-293
  - liberation of, 205
  - metabolism of, 300-305, 317
  - negative tests for, 287, 290
  - 3-oxo conjugate of, 300-301, 303, 305-314, 317, 320-323
  - paper chromatography of, 274, 288-290, 292, 295-299, 308-311
  - partition coefficient of, 294
  - potency ratio of, 270-272
  - purification of, 293-300
  - radioactive,
    - assay methods and, 284, 313-314, 330
    - excretion of, 300-304, 306, 323
    - secretion rate of, 305, 318-323
    - equation for, 319
  - tritiated, 279, 300, 313, 314, 316, 317, 319-322, 325, 330
- Aldosterone diacetate, 298
  - paper chromatography of, 292, 296, 310-311, 313
- Aldosteronism, urinary steroids in, 185-186, 322
- Alkali, adrenocorticosteroid fluorescence and, 252
  - aldosterone assay and, 278
- Allen equations,
  - adrenocorticosteroid assay and, 234, 237-238
  - aldosterone assay and, 276
  - pregnanediol assay and, 159
  - pregnanetriol assay and, 156-160
  - progesterone assay and, 112-115, 125, 129
- Allopregnane series, 141
- Allotetrahydrocortisol, 200
  - assay of, 243
- Alumina,
  - adrenaline assay and, 343-345, 347
  - adrenocorticosteroid assay and, 246
  - estrogen separation on, 12-13, 34-37
  - ketosteroid separation on, 57, 60, 72, 78, 80
  - pregnanediol isolation and, 149-150, 156
  - pregnanetriol isolation and, 151, 159-161
  - progesterone isolation and, 95, 101-102
- Alumina paper, progesterone isolation and, 103
- Amenorrhea, pregnanediol and, 163-164
- Ammonia,
  - estrogen separation and, 33, 34
  - thyroxine extraction and, 360
- Ammonium acetate, iodoamino acid separation and, 366
- Ammonium hydroxide, iodoamino acid separation and, 362, 364-366, 369

Ammonium ions, glucuronidase and, 206  
 Amniotic fluid, estrogens in, 44  
*tert.*-Amyl alcohol, iodoamino acid separation and, 366  
 Androstenediol, sulfuric acid and, 155  
 Androst-4-ene-3,17-dione,  
   daily secretion of, 52-53  
   metabolism of, 52  
   occurrence of, 143  
   partition coefficients of, 100  
   progesterone chromatography and, 129  
   separation of, 102, 105, 106  
 $\Delta^1$ -Androstene-3,17-dione, *see* Androst-4-ene-3,17-dione  
 5 $\alpha$ -Androst-16-en-3 $\alpha$ -ol,  
   determination of, 59-60  
   excretion of, 60  
 Androst-2(or 3)-en-17-one, determination of, 76  
 Androsterone, 63  
   adrenocorticosteroid assay and, 229  
   determination of, 77  
   9,11-epoxide of, 77  
   origin of, 52  
   partition chromatography of, 33  
   separation of, 72, 80  
   solvolytic of, 54  
 Aniline, iodine determination and, 372  
 Anovular bleeding, pregnanediol and, 163-164  
 Anthranilic acid, iodine determination and, 372  
 Antimony trichloride, progesterone identification and, 116  
 Appleby,  
   method, Burke modification of, 244  
   245  
 Arrhenoblastoma, virilism and, 142  
 Arsenite, iodine determination and, 359, 370-375  
 Arsenomolybdate, adrenaline assay and, 343  
 Ascites fluid, thyroxine binding in, 356  
 Ascorbic acid,  
   catechol amines and, 337, 339, 344-346  
   potassium hydroxide solution and, 70-71, 245  
 Autoradiography, iodoamino acid detection and, 364-365, 381  
 Ayres, method of, 278-280, 311, 313

## B

Bacteria, glucuronidase of, 205, 206  
 Barbiturate buffer, iodoamino acid electrophoresis and, 355  
 Bartter, method of, 309, 329-330  
 Baulieu and De Vigan, method of, 309  
 Benedict's reagent, 110  
 Benzalkonium chloride, noradrenaline assay and, 343  
 Benzene,  
   adrenocorticosteroid isolation and, 212, 214, 228, 231, 235, 238, 246  
   aldosterone isolation and, 288-289, 292-294, 296, 309, 311, 317  
   estrogen isolation and, 8, 9, 11, 13, 16, 46, 62  
   ketosteroid isolation and, 57, 60, 61, 65, 66, 72, 78-80  
   pregnanediol isolation and, 156-158  
   pregnanetriol isolation and, 151, 159-160  
   pregnanetriolone isolation and, 173, 177  
   progesterone isolation and, 95, 97, 102, 104, 105, 128-129  
   tetrahydroaldosterone isolation and, 316  
 Bile,  
   estrogens in, 43-44  
   pregnanediol in, 144  
 3,3'-Bis[4,4'-(3,5-diphenyl)tetrazolium chloride]. *See* Blue tetrazolium  
 Bismuthate,  
   adrenocorticosteroids and, 215, 217, 240-247  
   pregnanetriol and, 151  
   pregnanetriolone assay and, 180  
   purity, reaction time and, 243  
 Bisulfite,  
   acetaldehyde estimation and, 179  
   iodine determination and, 359  
 Blood, *see also* Plasma  
   adrenal vein, aldosterone in, 328-331  
   adrenocorticosteroids in, 202-204  
   aldosterone in, 308-311, 316, 323-327  
   pregnanediol in, 144  
   progesterone in, 119, 120  
 Blue tetrazolium, *see also* Formazan  
   adrenocorticosteroids and, 215, 216, 218-224, 252

- aldosterone assay and, 276-278, 290, 299, 308-309
- aldosterone metabolites and, 302, 307, 315
- Bojesen and Degn, method of, 311, 324, 330
- Bradosol, adrenocorticosteroid isolation and, 210-211
- Brooks, method of, 310
- Brooksbank and Haslewood, method of, 59-60
- Brucine sulfate, iodine determination and, 372, 375
- Burstein and Lieberman, methods of, 53-56
- Bush,  
method, Gowenlock application of, 295
- Bush and Sandberg, method of, 295
- Butanol,  
adrenocorticosteroid assay and, 233, 236-237  
adrenocorticosteroid conjugates and, 209  
aldosterone isolation and, 288-289, 296  
estrogen separation and, 31, 33, 34  
iodoamino acid separation and, 361-362, 364-367, 369  
pregnanediol isolation and, 148  
thyroxine extraction by, 359-360
- sec*-Butanol, iodoamino acid separation and, 362
- tert*-Butanol,  
aldosterone isolation and, 288-289, 296  
iodoamino acid separation and, 362
- Butt, method of, 88, 119, 120
- Butyl acetate, aldosterone isolation and, 288-289, 296, 309
- Butyl ether, estrogen separation and, 33
- Calcium carbonate, pregnanediol isolation and, 149
- Callow, method of, 84
- Carbon disulfide, iodine determination and, 370
- Carbon tetrachloride,  
adrenocorticosteroid isolation and, 213, 232, 239  
estrogen separation and, 15, 16, 37  
ketosteroid isolation and, 69, 72  
pregnanetriolone isolation and, 177
- Casein, iodination of, 378-379
- Catechol acids, catechol amine assay and, 344, 347
- Catechol amines, *see also specific compounds*  
adrenocorticosteroid assay and, 219
- Celite,  
aldosterone isolation and, 310-311, 317, 325  
aldosterone metabolite and, 315, 316  
estrogen separation on, 16, 32, 33, 36, 37  
pregnanediol isolation and, 149
- Cellana tramoserica*, glucosidase of, 173
- Cellulose powder, iodoamino acid separation and, 362
- Cerebrospinal fluid, thyroxine-binding in, 356
- Ceric ions, iodine determination and, 370-375
- Chloral hydrate, adrenocorticosteroid assay and, 232
- Chloric acid, iodine determination and, 359
- Chloride,  
adrenocorticosteroid assay and, 241  
iodine determination and, 370-371, 373
- $3\beta$ -Chloroandrost-5-ene-17-one, determination of, 76
- Chloroform,  
adrenocorticosteroid isolation and, 207-208, 210, 229, 238  
aldosterone isolation and, 288-289, 293, 294, 296-300, 305-306, 308-312, 317, 324, 325, 328  
cortisol isolation and, 300  
estrogen separation and, 11, 16, 31, 37, 46  
iodine determination and, 352  
iodoamino acid separation and, 361, 364  
ketosteroid isolation and, 61, 81, 83, 84  
pregnanetriolone isolation and, 173, 175, 176  
progesterone isolation and, 101, 128  
thyroxine extraction and, 360
- Chloroquinone imines, iodoamino acids and, 378
- Cholestenone, thiosemicarbazone of, 108
- Cholesterol, corticosteroids and, 143

- ketosteroids and, 59
- sulfuric acid and, 155
- Chromatography, principles of, 296-298
- Chromic acid,
  - aldosterone and, 290-291, 311, 314, 324
  - iodine determination and, 359
  - pregnanetriol assay and, 161
  - pregnanetriolone assay and, 180
- Chromosorb, estrogen separation on, 18
- Chromotropic acid, adrenocorticosteroids and, 215, 225
- Cirrhosis, aldosterone and, 304
- Citrate, iodine determination and, 373
- Citrus fruits, steroid in, 307, 312
- Colchicine, adrenocorticosteroid assay and, 232
- Collidine, iodoamino acid separation and, 366, 372
- Conn's syndrome,
  - aldosterone metabolism in, 304, 305, 321
  - urinary steroids in, 182, 185, 186
- Coppage, method of, 315
- Copper, aldosterone assay and, 281
- Copper acetate, thyroxine and, 379
- Corpus luteum,
  - hormone of, 91-92
  - pregnanediol and, 161-163
  - progesterone in, 119, 120, 130-132
- Corticosterone,
  - assay of, 248-249
  - blue tetrazolium and, 220
  - extraction of, 328
  - isolation of, 212, 214, 248
  - phenylhydrazine and, 232-233
  - precursors of, 143
  - recovery of, 250
  - tritiated, metabolism of, 320
- Corticosterone acetate, paper chromatography of, 292
- Cortisol, 170, 315
  - assay of, 248, 249, 293
  - biosynthesis of, 189
  - blue tetrazolium and, 220, 221
  - daily secretion of, 52-53
  - dinitrophenylhydrazine and, 281
  - extraction of, 307
  - fluorescence of, 174, 177-179, 248, 252
  - isolation of, 212-214, 248, 295
  - metabolism of, 52
  - paper chromatography of, 288-289, 297, 312
  - partition coefficient of, 294
  - periodate and, 226
  - phenylhydrazine and, 231, 232, 235, 236
  - pregnanetriolone formation and, 194, 195
  - radioactive, 282
    - metabolism of, 254-255, 300-301
  - sodium excretion and, 267-268, 270, 274
  - urinary, 316
- Cortisol acetate, paper chromatography of, 313
- Cortisone,
  - alkali and, 212
  - destruction of, 242
  - dinitrophenylhydrazine and, 281
  - extraction of, 307
  - isolation of, 248
  - paper chromatography of, 288-289, 297, 312
  - periodate and, 226
  - phenylhydrazine and, 231, 234, 235
  - pregnanetriol and, 144
  - pregnanetriolone excretion and, 188, 194
  - sodium excretion and, 267-268, 270, 274
- Cortisone acetate,
  - blue tetrazolium and, 219
  - fluorescence of, 252
  - paper chromatography of, 313
- Cortol, occurrence of, 200
- Cortolone(s),
  - assay of, 225
  - occurrence of, 200
- $\beta$ -Cortolone,
  - alkali and, 212
  - isolation of, 208
  - occurrence of, 200
- Cox, method of, 179-180, 229-230
- Cox and Finkelstein, method of, 173-176
- Creatinine, urine collection and, 204
- Cushing's syndrome, 201
  - adrenals and, 170
  - urinary steroids in, 184, 186, 189-191, 195-196, 251
- Cyclohexane,
  - aldosterone isolation and, 288-290, 292-294, 296, 311
  - estrogen separation and, 11, 15

- iodoamino acid separation and, 362  
 progesterone isolation and, 105  
 tetrahydroaldosterone isolation and, 315
- Cystine, iodine determination and, 373
- ### D
- Das Gupta and Giroud, method of, 308, 329
- Davis, method of, 311, 330
- Decolorite, estrogen separation and, 33
- 11-Dehydrocorticosterone acetate, paper chromatography of, 292
- Dehydroepiandrosterone, 63  
 adrenocorticosteroid assay and, 241, 242  
 daily secretion of, 52-53  
 determination of, 53-59  
 metabolism of, 52  
 partition chromatography of, 33  
 separation of, 72, 80  
 standard solutions of, 57
- Dehydroisoandrosterone, adrenocorticosteroid assay and, 228, 229
- 11-Dehydrocorticosterone, isolation of, 212  
 phenylhydrazine and, 232-233
- $\Delta^{11}$ -Dehydropregesterone, separation of, 103
- Delsal's reagent, estrogens and, 43, 45
- Deming and Luetscher, method of, 269
- Deoxycorticosterone, partition coefficients of, 100  
 precursors of, 143  
 pregnanediol and, 140, 142  
 progesterone and, 142  
 separation of, 105  
 sodium excretion and, 267-270, 272
- 11-Deoxycortisol, blue tetrazolium and, 219  
 daily secretion of, 52-53  
 metabolism of, 52  
 pregnanetriolone formation and, 194
- 21-Deoxycortisol, pregnanetriolone and, 193, 194
- Deoxycortisone, dinitrophenylhydrazine and, 280  
 paper chromatography of, 292  
 pregnanetriolone and, 193
- Diabetes, adrenocorticosteroid assay and, 232
- Diatrizoate, protein and, 357
- Diazotized sulfanilic acid, estrogen estimation by, 19  
 iodoamino acids and, 377, 378
- Dibromotyrosine, 377
- o*-Dichlorobenzene, estrogen separation and, 11
- Dichloromethane, *see also* Methylene chloride, Methylene dichloride  
 adrenocorticosteroid isolation and, 211, 253  
 aldosterone isolation and, 294  
 ketosteroid determination and, 66  
 pregnanetriol isolation and, 160
- Dichlorotyrosine, 377
- Diet, aldosterone and, 307
- N', N'-Diethylsulfanilamide, iodoamino acids and, 377-378
- Digitonin, ketosteroids and, 86-88
- 20,21-Dihydroxy-18,20-epoxy-4-pregnen-3-one, phenylhydrazine and, 233
- Dihydroxyphenylalanine, noradrenaline assay and, 343, 346
- Dihydroxyphenylethylamine, noradrenaline assay and, 343
- 11 $\beta$ ,21-Dihydroxypregna-4,16-diene-3, 20-dione, phenylhydrazine and, 232-233
- 3 $\alpha$ ,17 $\alpha$ -Dihydroxy-5 $\beta$ -pregnane-11,20-dione, pregnanetriolone and, 193  
 recovery of, 242
- 3 $\beta$ ,16 $\alpha$ -Dihydroxypregnan-20-one, sodium excretion and, 275
- 17,20-Dihydroxysteroids, assay of, 225, 230
- 3,3'-Diiodothyroacetic acid, paper chromatography of, 366
- "3,5"-Diiodothyroacetic acid, paper chromatography of, 366
- Diiodothyronine(s), ninhydrin and, 375-376
- 3,5-Diiodothyronine, arsenite-ceric reaction and, 370-371, 373-374  
 column chromatography of, 363  
 occurrence of, 353  
 paper chromatography of, 366  
 ultraviolet spectroscopy of, 379
- 3,3'-Diiodothyronine,

- desiccated thyroid and, 380  
occurrence of, 353  
paper chromatography of, 365-366
- Diiodotyrosine,  
arsenite-ceric reaction and, 370-371,  
373-374  
column chromatography of, 361-363  
coupling of, 352, 353  
diazotized diethylsulfanilamide and,  
378  
extraction of, 359-360  
iodinated proteins and, 369  
isolation of, 352  
ninhydrin and, 375-376  
nitrous acid and, 376  
paper chromatography of, 364-367  
Pauly's reagent and, 377  
polarography of, 380  
radioactive, 380-381  
ultraviolet spectroscopy of, 378, 379
- m*-Dinitrobenzene, 110, *see also* Zimmermann reaction  
adrenocorticosteroids and, 245, 246  
ketosteroids and, 52, 56, 64, 66-68, 84  
progesterone and, 119  
purification of, 70
- 2,4-Dinitrophenylhydrazine,  
adrenocorticosteroids and, 256  
aldosterone assay and, 280-281, 309  
cortisol and, 281  
cortisone and, 281  
deoxycortisone and, 280  
oxidation of, 110  
progesterone derivative, 95, 102, 109-110, 116, 117, 119, 134
- Diodrast, protein and, 357
- Dioxane,  
aldosterone isolation and, 288-290, 292, 296, 311  
estrogen hydrolysis by, 6, 31  
iodoamino acid separation and, 367  
steroid extraction and, 208
- Diphenyl oxazole, aldosterone assay and, 285
- Diphosphopyridine nucleotide, estrogen estimation and, 21-22
- Dithionite, ketosteroid determination and, 69
- Dog, aldosterone assay and, 269-270
- Dorfman, method of, 267
- Dowex-1, iodoamino acid separation on, 362, 363, 364
- Dowex-50, catechol amine assay and, 347
- Drekter, method of, 64-65
- Dyrenfurth and Venning, method of, 309, 312
- E**
- Edgar, method of, 119, 120
- Edwards and Kellie, method of, 245-247
- Electrophoresis, iodoamino acids and, 355-357
- Elliott, Birmingham, Schally and Schönbau, method of, 223-224
- Emulsions, breaking of, 210-211
- Enovid, aldosterone metabolism and, 304, 321
- Enzymes,  
adrenocorticosteroid conjugates and, 205-207  
estrogen conjugates and, 6-7, 32, 45  
estrogen estimation by, 21-22  
ketosteroid hydrolysis by, 53  
pregnanetriol and, 150, 154
- Epiandrosterone, solvolysis of, 54
- 16-Epiestriol,  
chromatography of, 46  
occurrence of, 3  
stability of, 45  
structure of, 2
- Epitestosterone, separation of, 105
- Equilenin, occurrence of, 2
- Equilin, occurrence of, 2
- Erepsin, iodoamino acid liberation and, 369
- Estradiol-17 $\beta$ ,  
estimation, 35-40  
chromatography and, 9-18  
extraction and, 7-9  
hydrolysis and, 5-7  
purification and, 9  
quantitation and, 18-22  
sample collection and, 4-5  
structure of, 2  
testosterone determination and, 61-63
- Estradiol-17 $\beta$  glucosiduronate, fluorometry of, 34
- Estriol,  
estimation, 35-40  
chromatography and, 9-18, 28

- extraction and, 7-9
- hydrolysis and, 5-7
- purification and, 9
- quantitation and, 18-22
- sample collection and, 4-5
- separation of, 8
- solvent partition of, 31
- structure of, 2
- Estriol-16 $\beta$ , 17 $\beta$ , *see* 16-Epicestriol
- Estriol glucosiduronate,
  - fluorometry of, 34
  - solvent partition of, 31, 33
- Estrogens, *see also specific compounds*
  - adsorption column chromatography of, 12-14, 26, 35
  - aldosterone binding and, 327
  - anabolic pathway of, 142-143
  - bioassay of, 29-30
  - column partition chromatography of, 14, 16-18, 23-27, 37-40
  - conjugates, 3-4
    - chromatography of, 32-34
    - differential hydrolysis of, 31-32
    - estimation of, 30-34
    - hydrolysis of, 5-7
    - quantitation of, 34
    - solvent partition of, 30-31
  - countercurrent distribution of, 14-15, 17, 24, 26, 27, 33, 43
  - definition of, 1-2
  - derivatives, chromatography of, 27-28
  - estimation,
    - criteria for, 4
    - difficulties of, 3-4
    - routine, 35-40
  - extraction of, 23-24, 44
  - gas chromatography of, 18
  - hydrolysis of, 23-24, 45
  - identification of, 25-30, 40-41
  - infrared spectroscopy of, 28-29, 43
  - ion exchange chromatography of, 14
  - isotopes and, 22-25
  - multiple chromatography of, 26-27
  - paper chromatography of, 10-12, 26, 27, 32, 43, 45-46
  - proteins and, 3-4, 43-45
  - structure of, 2
- Estrone,
  - estimation, 35-40
  - chromatography and, 9-18, 28
  - extraction and, 7-9
  - hydrolysis and, 5-7
  - purification and, 9
  - quantitation and, 18-22
  - sample collection and, 4-5
  - structure of, 2
  - testosterone determination and, 61
- Estrone sulfate,
  - chromatography of, 33
  - fluorometry of, 34
- Ethanol,
  - adrenocorticosteroid isolation and, 209-211, 213, 231, 244, 246, 248, 253
  - aldosterone assay and, 286, 288-289, 294, 296, 299, 310
  - estrogen isolation and, 13, 15, 43, 44
  - iodoamino acid separation and, 364
  - ketosteroid determination and, 69, 72, 78, 80
  - pregnanediol isolation and, 148, 149, 156-157, 159
  - pregnanetriol isolation and, 159-160
  - progesterone isolation and, 94-96, 100-101, 103, 113, 124, 128
  - purification of, 222, 229
- Ether,
  - adrenocorticosteroid assay and, 207, 214, 226-228, 244-246
  - aldosterone isolation and, 293, 309
  - estrogen isolation and, 8, 13, 30-33
  - ketosteroid extraction by, 53, 60, 61, 64, 68, 75, 79-81, 84-86
  - pregnanediol isolation and, 149
  - pregnanetriol isolation and, 159
  - pregnanetriolone isolation and, 177
  - progesterone extraction by, 94-96, 98-99, 124, 127
- Ethyl acetate,
  - adrenocorticosteroid isolation and, 208, 209, 211, 212, 227
  - aldosterone isolation and, 288-289, 294, 295, 296, 301, 308-309, 311
  - estrogen separation and, 13, 15, 31, 33
  - ketosteroid isolation and, 53-55, 82
  - pregnanediol isolation and, 158
  - progesterone extraction and, 95, 97, 124
  - tetrahydroaldosterone isolation and, 314-316
- Ethylenediamine, catechol amine assay and, 339-341, 345-347



- Ethylene dichloride (Ethylene chloride),  
adrenocorticosteroid isolation and, 208  
aldosterone metabolite and, 315  
estrogen separation and, 16, 33  
ketosteroids and, 64, 83  
noradrenaline assay and, 343  
pregnanetriolone isolation and, 173, 175  
tetrahydroaldosterone isolation and,  
315
- Ethyl glycol, aldosterone isolation and,  
288-289, 309
- Ethyl oleate, aldosterone isolation and,  
311, 324
- Ethinylestradiol 3-methyl ether, aldosterone binding and, 327
- Etiocholanolone, 63  
adrenocorticosteroid assay and, 229  
determination of, 77  
isolation of, 242  
origin of, 52  
separation of, 72, 80
- Etiocholanolone acetate, determination of, 76
- F**
- Farrell, method of, 310, 329
- Farrell and Richards, method of, 268-269, 271
- Fat, progesterone metabolites in, 145
- Fat tissue, progesterone in, 119, 130, 132
- Fatty acids, adrenocorticosteroid assay and, 220
- Feces,  
estrogens in, 44  
pregnanediol in 144-145, 165
- Fehling's solution, 110
- Ferric chloride, iodine determination and, 372-373
- Ferrieyanide,  
catechol amino assay and, 344, 345  
iodine determination and, 372-373
- Ferrioin, iodine determination and, 372
- Ferrous ammonium sulfate, iodine determination and, 375
- Ferrous sulfate, iodine determination and, 372
- Fertility, pregnanediol and, 163
- Finkelstein, method of, 60-63, 176-177
- Finkelstein and Goldberg, method of, 177-179
- Flood, method of, 278, 310, 313, 315-316, 322-323, 326
- Florisil,  
adrenocorticosteroid isolation and, 212-214, 235, 238-239, 249  
ketosteroid separation on, 80-84  
pregnanetriol isolation and, 151
- Fluorescein, iodine determination and, 372
- Fluorescence,  
catechols and, 338  
adrenocorticosteroids and, 215, 247-254  
aldosterone assay and, 278-280, 293, 299, 307-311, 313, 317, 328-330  
pregnanetriol assay and, 161  
progesterone identification and, 116, 119, 133-134
- Fluorometry,  
estrogen estimation by, 20-21, 34, 37, 46, 62  
pregnanetriolone assay and, 172, 177-179  
progesterone estimation and, 111-112
- Folin and Ciocalteu's reagent, estrogen estimation by, 19
- Follicular fluid,  
hormones in, 143  
progesterone in, 119
- Formaldehyde, adrenocorticosteroids and, 215, 216, 225-228
- Formamide,  
adrenocorticosteroids and, 228  
aldosterone isolation and, 288-289, 293, 296, 297, 308-312  
estrogen separation and, 11, 46  
pregnanetriolone isolation and, 173, 175  
progesterone isolation and, 105, 106  
tetrahydroaldosterone isolation and, 315, 324
- Formazan, elution from paper, 22, 315
- Formic acid, iodoamino acid separation and, 363, 365
- Fotherby, method of, 56-57
- Frame, method of, 87
- Furfural, ketosteroid determination and, 57-59
- G**
- Ganong, method of, 308, 329
- Gardner, method of, 83-84

- Garst, method of, 310  
 Girard's reagent,  
   adrenocorticosteroids and, 227-229  
   ketosteroid determination and, 67, 72,  
     75, 85  
   pregnanetriol isolation and, 161  
   progesterone and, 94, 99-100, 107-108  
 Glass fiber paper, aldosterone isolation  
   and, 299  
 Globulins, iodoamino acids and, 354-356  
 Glucocorticoids, sodium excretion and,  
   270, 271  
 Glucose, adrenocorticosteroid assay and,  
   240, 244  
 Glucuronic acid,  
   pregnanediol assay and, 148  
   thyroxine and, 365  
 $\beta$ -Glucuronidase, 312  
   adrenocorticosteroids and, 205-207, 227,  
     229, 231, 238, 239  
   aldosterone and, 300, 301-303, 309  
   cortisol and, 300  
   estrogens and, 6, 32  
   inhibition of, 206  
   ketosteroids and, 60, 79  
   pregnanediol assay and, 149, 158  
   pregnanetriol and, 145, 151, 159, 160  
   pregnanetriolone assay and, 173  
   tetrahydroaldosterone isolation and,  
     314, 315, 321  
   thyroxine conjugates and, 365  
 Glucosiduronates, hydrolysis of, 6  
 Glycerol, adrenocorticosteroid assay and,  
   243  
 Glycols, adrenocorticosteroid assay and,  
   220, 243  
 Glycoproteins, iodoamino acids and, 356  
 Gorski, method of, 119, 121  
 Gowenlock, method of, 311  
 Graafian follicle, progesterone in, 130-131  
 Graves disease, thyroxine in, 364
- ## H
- Hamburger and Rasch, method of, 65  
 Haskins, method of, 119, 120  
 Heart, thyroxine binding in, 356  
 Helium, estrogen separation and, 18  
 Heparin, plasma collection and, 54  
 Hepatitis, aldosterone and, 304  
 Heptane,  
   ketosteroid separation and, 75-77  
   progesterone isolation and, 106  
 Heptanol, aldosterone isolation and, 295-  
   296, 309  
 Hermaphroditism, pregnanetriolone and,  
   181, 182  
 Hexane,  
   aldosterone isolation and, 292, 309-310  
   estrogen separation and, 15, 16, 33, 37  
   progesterone isolation and, 100, 101,  
     102, 103, 113, 125, 128-129  
 Hilus-cell tumor, ketosteroids and, 63  
 Hinsberg, method of, 119, 120  
 Hirsutism,  
   ketosteroids and, 59  
   pregnanetriolone and, 181  
 Holtorf and Koch, method of, 84, 85  
 Human,  
   aldosterone metabolism in, 300-305  
   estrogens of, 2  
 Hydrocortisone, formation of, 143  
 17-Hydroxyandrostanes, oxidation of, 241  
 11 $\beta$ -Hydroxyandrost-4-ene-3,17-dione,  
   daily secretion of, 52-53  
   metabolism of, 52  
   separation of, 105  
 3 $\alpha$ -Hydroxy-5 $\alpha$ -androst-9(11)en-17-one,  
   oxidation of, 77  
 3 $\alpha$ -Hydroxy-5 $\beta$ -androst-9(11)en-17-one,  
   formation of, 243, 244  
   oxidation of, 77  
 11-Hydroxyandrosterone, 52, 63  
   adrenocorticosteroid assay and, 229  
   separation of, 80  
   stability of, 72  
 17-Hydroxycorticosterone, formation of,  
   143  
 18-Hydroxycorticosterone,  
   aldosterone biosynthesis and, 328  
   metabolite of, 302  
 6 $\beta$ -Hydroxy-3,5-cycloandrostan-17-one,  
   chromatography of, 57  
 17-Hydroxydeoxycorticosterone, forma-  
   tion of, 143  
 18-Hydroxydeoxycorticosterone, 290, 315  
 18-Hydroxydeoxycortisone, phenylhy-  
   drazine and, 233  
 4-Hydroxydiphenyl,  
   acetaldehyde estimation and, 179  
   adrenocorticosteroid assay and, 229-230

- 16 $\alpha$ -Hydroxyvestrone,  
  chromatography of, 46  
  fluorescence of, 46  
  occurrence of, 3  
  structure of, 2
- 16 $\beta$ -Hydroxyvestrone,  
  occurrence of, 3  
  solubility of, 45  
  structure of, 2
- 18-Hydroxyestrone,  
  occurrence of, 3  
  structure of, 2
- 11-Hydroxyetiocholanolone, 52, 63  
  adrenocorticosteroid assay and, 228  
  formation of, 242  
  separation of, 80  
  stability of, 72
- 5-Hydroxyindoles, adrenocorticosteroid  
  assay and, 219
- Hydroxylamine, aldosterone metabolites  
  and, 291, 302
- Hydroxylases, pregnanetriolone forma-  
  tion and, 194-195
- 20-Hydropregnanes, oxidation of, 241
- 17 $\alpha$ -Hydroxyprogesterone,  
  daily secretion of, 52-53  
  formation of, 143  
  metabolism of, 52  
  occurrence of, 143  
  partition coefficients of, 100  
  pregnanetriol and, 140-142, 146-147,  
    150-151  
  pregnanetriolone and, 194, 195  
  separation of, 105
- 3 $\beta$ -Hydroxy-5 $\alpha$ -pregnan-20-one, thiosemi-  
  carbazone of, 108
- 20 $\alpha$ -Hydroxypregn-4-en-3-one,  
  formation of, 117  
  partition coefficients of, 100  
  separation of, 105, 106
- 20 $\beta$ -Hydroxypregn-4-en-3-one,  
  formation of, 117  
  partition coefficients of, 100  
  separation of, 105, 106
- Hydroxysteroid dehydrogenase(s), estro-  
  gen estimation by, 21
- 20 $\alpha$ -Hydroxysteroid dehydrogenase, pro-  
  gesterone identification and, 117
- 20 $\beta$ -Hydroxysteroid dehydrogenase, pro-  
  gesterone identification and, 117
- Hyflo Supercel,  
  iodoamino acid separation on, 361  
  progesterone isolation on, 93-95, 102
- Hypaque, protein and, 357
- Hypertension, aldosterone metabolism  
  and, 304, 320, 322
- Hyperthyroidism, triiodothyronine up-  
  take in, 356
- Hypertrichosis, *see also* Hirsutism  
  pregnanediol and, 164, 165
- Hypogonadism, testosterone and, 53
- Hypopituitarism, 201
- Hypothyroidism, triiodothyronine uptake  
  in, 356
- I
- Immunoelectrophoresis, iodoamino acids  
  and, 356
- Incineration, iodine determination and,  
  358-359
- Infrared spectroscopy,  
  aldosterone identification and, 287, 293  
  estrogens and, 28-29, 43  
  progesterone identification and, 117-119
- Insulin, iodination of, 379
- Iodate, iodine determination and, 370
- Iodeikon, protein and, 357
- Iodide, adrenocorticosteroid assay and,  
  232  
  column chromatography of, 361-363  
  extraction of, 359  
  paper chromatography of, 366  
  radioactive, 380
- Iodine,  
  catechol amine assay and, 342, 344  
  determination of, 352, 370-375  
  distillation of, 359  
  20-hydroxypregnenone isomers and, 106
- Iodoamino acids,  
  binding of, 354  
  free,  
    column chromatography of, 361-363  
    determination of, 369-382  
    electrophoresis of, 355-357  
    paper chromatography of, 363-367,  
      381  
    protein-bound iodine procedure and,  
      357-359  
    solvent extraction of, 359-361

- peptide-bound,  
determination of, 369-382  
hydrolysis of, 368-369  
isolation of, 367-368  
separation of, 369
- p*-Iodobenzenesulfonyl chloride, aldosterone assay and, 282-285, 311, 324, 330
- Iodo-histidine, iodinated proteins and, 369
- 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, adrenocorticosteroids and, 218-219
- Iodopyracet, protein and, 357
- 3-Iodothyronine, ultraviolet spectroscopy of, 379
- Ion exchange resins, estrogen purification and, 9, 14
- Iophenoxic acid, protein and, 357
- 17-Isoaldosterone, aldosterone assay and, 312-313
- Isobutanol, catechol amine assay and, 347
- Iso-citric dehydrogenase, estrogen estimation and, 22
- Isonicotinic acid hydrazine, progesterone derivative, 95, 102, 110, 117
- Isocetane,  
aldosterone isolation and, 288-289, 295  
estrogen separation and, 11, 46  
pregnanediol isolation and, 158
- Isopentanol, iodoamino acid separation and, 365-366
- Isopropanol, estrogen separation and, 13
- Isopropyl acetate, adrenocorticosteroid isolation and, 208
- Isopropyl ether, estrogen separation and, 33
- Isotopes,  
adrenocorticosteroids and, 218, 254-256  
aldosterone assay and, 282-286, 298, 330-331  
estrogen estimation and, 24-25, 43  
iodoamino acids and, 380-382  
testosterone determination and, 61-63
- Izzo, Keutmann and Burton, method of, 222-223
- J**
- Jaundice, adrenocorticosteroid assay and, 232
- Johnson, method of, 271
- Joint fluid, thyroxine binding in, 356
- Jones, method of, 317-318, 322
- K**
- Kagawa, method of, 269, 271
- Kaolin, adrenocorticosteroid assay and, 233
- Kellie and Wade, method of, 79-80
- 11-Ketoandosterone, 52, 63  
adrenocorticosteroid assay and, 229  
determination of, 78  
separation of, 80
- Ketodase,  
aldosterone metabolites and, 314, 316  
estrogen conjugates and, 6, 32  
pregnanediol assay and, 158  
pregnanetriolone isolation and, 173
- 11-Keto- $\Delta^1$ -cholestanolone, 52, 63  
adrenocorticosteroid assay and, 228  
cortolone assay and, 225  
determination of, 78  
formation of, 242  
separation of, 80
- 11-Keto-pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol, *see* Pregnanetriolone
- 11-Ketoprogesterone, separation of, 105
- $\Delta^1$ -3-Ketosteroids, phenylhydrazine and, 231
- 17-Ketosteroids, *see also specific compounds*  
adrenocorticotrophic hormone and, 181  
assay of, 63-72  
beta fraction of, 86-88  
chromatography of, 55-56, 82-83  
components, 52  
fractionation of, 72-80  
gradient elution chromatography of, 78-80  
miscellaneous methods for, 88  
neutral ketonic fractions and, 85-86  
origin of, 52-53  
paper chromatography of, 73-78, 80  
virilism and, 53, 59, 63, 170, 192-193
- Kidney,  
adrenocorticosteroids and, 203  
estrogens in, 43  
iodoamino acids in, 362  
thyroxine binding in, 356

- Kieselguhr**,  
  aldosterone isolation and, 317, 325  
  aldosterone metabolite isolation and, 316  
  estrogen separation on, 16  
  iodoamino acid separation on, 361-362  
**Klendshoj**, method of, 67  
**Kliman and Peterson**, method of, 311  
  313-314, 330  
**Kober reaction**,  
  estrogen estimation by, 19-20, 35-36, 42, 46  
  pregnanetriol assay and, 161  
  sensitivity of, 153
- L**
- Lakshmann and Lieberman**, method of, 78  
**Leucoadrenochrome**, formation of, 338-339  
**Lewbart and Mattox**, method of, 281  
**Liddle**, method of, 269-271, 277-278  
**Lieberman and Leutscher**, method of, 308  
**Light petroleum**, *see also* Petroleum ether  
  estrogen extraction by, 8  
  ketosteroid separation and, 81  
  progesterone isolation and, 99, 103, 104, 127  
**Ligroin**, *see also* Petroleum ether  
  ketosteroid separation and, 61  
  progesterone isolation and, 125  
  tetrahydroaldosterone isolation and, 315  
**Lipids**, freezing out, 97, 124, 128  
**Lipoproteins**, iodoamino acids and, 356  
**Liver**,  
  adrenocorticosteroids and, 203  
  estrogens in, 43, 44  
  glucuronidase of, 205  
  iodoamino acids in, 362  
  ketosteroids and, 59  
  pregnanediol and, 146  
  thyroxine binding in, 356  
**Loy**, method of, 119, 120  
**Lutines**, adrenaline assay and, 338-339  
**Lymph**, thyroxine binding in, 356
- M**
- Manganese dioxide**, catechol amine assay and, 344  
**Marcus**, method of, 268, 271  
**Mattox and Lewbart**, method of, 309  
**Meconium**, estrogens in, 43-44  
**Medical Research Council**, method of, 67-72  
**Menstrual cycle**, 2  
  pregnanediol and, 161-163  
  progesterone in, 132  
**Meprobamate**, adrenocorticosteroid assay and, 243  
**Mercury**, iodine determination and, 370-371  
**Methanol**,  
  adrenocorticosteroid isolation and, 213, 231, 238  
  aldosterone isolation and, 288-289, 292, 295, 296, 298, 308-311, 317, 325, 328  
  estrogen separation and, 11, 13, 15, 16, 43, 46, 62  
  iodoamino acid separation and, 366, 369  
  ketosteroid separation and, 55, 56, 74, 81, 83  
  pregnanediol isolation and, 149, 158  
  pregnanetriolone isolation and, 173, 177  
  pregesterone isolation and, 94-95, 97, 99, 100, 102-104, 125, 127-129  
  purification of, 229  
  testosterone determination and, 61  
  tetrahydroaldosterone isolation and, 315, 316  
**Methionine**, iodine determination and, 373  
**2-Methoxyestriol**  
  occurrence of, 3  
  structure of, 2  
**2-Methoxyestrone**,  
  fluorescence of, 46  
  Kober reaction and, 46  
  occurrence of, 3  
  solubility of, 45  
  structure of, 2  
**Methylal**, estrogens and, 43  
**Methyl cellosolve**, ketosteroid determination and, 67  
**Methylcyclohexane**,  
  aldosterone metabolite and, 315  
  progesterone isolation and, 104, 128, 129  
**Methylene blue**, iodine determination and, 372

- Methylene chloride, *see also* Dichloromethane, Methylene dichloride  
 estrogen separation and, 11, 13  
 ketosteroid separation and, 55  
 pregnanediol isolation and, 158, 159  
 progesterone isolation and, 97, 99, 102
- Methylene dichloride, *see also* Dichloromethane, Methylene chloride  
 adrenocorticosteroid isolation and, 208, 210, 214, 229, 232, 238, 239, 244, 246, 249, 253  
 aldosterone isolation and, 294, 307, 311, 330  
 purification of, 223  
 tetrahydroaldosterone isolation and, 315, 316
- Methyl ethyl ketone, estrogen separation and, 33
- O-Methyl thyroxine, paper chromatography of, 366
- 1-Methyl-3,5,6-trihydroxyindole, formation of, 338
- Miescher reaction, ketosteroids and, 60
- Migeon and Plager, method of, 80, 81
- Millon's reagent, iodoamino acids and, 376
- Monochlorobenzene, estrogen separation and, 11, 46
- Monoiodotyrosine,  
 arsenite-ceric reaction and, 373  
 column chromatography of, 361-363  
 formation of, 360  
 iodinated proteins and, 369  
 Millon's reagent and, 376  
 ninhydrin and, 375-376  
 paper chromatography of, 364-367  
 radioactive, 380-381  
 tumor proteins and, 368
- Moolenaar, method of, 280-281, 309
- Mulrow, method of, 308, 329
- Munson, method of, 57-59
- Muscle, thyroxine binding in, 356
- Muscle fatigue, aldosterone and, 266
- Mylase, estrogen conjugates and, 7, 32
- N**
- 1,2-Naphthoquinone-4-sulfonate, noradrenaline and, 341, 343
- Naphthoresorcinol reaction,  
 estrogen glucosiduronates and, 34  
 pregnanediol assay and, 148
- Neher and Wettstein, method of, 295, 298, 307-313, 329
- Nelson and Samuels,  
 method, Eik-Nes modification of, 238-239
- Ninhydrin, iodoamino acids and, 375
- p*-Nitrophenol, estrogen estimation and, 20
- Nitrous acid, iodoamino acid determination and, 376
- Noradrenaline, *see also*  
 chemistry of, 337-338  
 ethylenediamine and, 341  
 fluorescence of, 338  
 iodine and, 342  
 permanganate and, 342-343
- Nowaczynski, method of, 277, 309, 312
- O**
- Octanol, aldosterone isolation and, 309
- Oertel, method of, 119, 121, 128-130
- Osmium, iodine determination and, 370
- Ovary, 2  
 hormone of, 92  
 pregnanetriol and, 142-143  
 pregnanetriolone and, 193
- Oxalate, plasma collection and, 54
- Oxalic acid, catechol amine assay and, 344
- 16-Oxoestradiol-17 $\beta$ ,  
 chromatography of, 46  
 fluorescence of, 46  
 occurrence of, 3  
 structure of, 2
- 16-Oxoestrone, fluorescence of, 46
- P**
- Pancreatin, iodoamino acid liberation and, 369
- Paraldehyde, adrenocorticosteroid assay and, 232
- Patella vulgata*, 79  
 estrogen conjugates and, 7
- Pauly's reagent, iodoamino acids and, 377
- Pencillin G, ketosteroid hydrolysis and, 79
- Perbenzoic acid, ketosteroid determination and, 77
- Periodate,  
 adrenocorticosteroids and, 215-217, 224-230

- aldosterone and, 291  
pregnanetriol assay and, 151, 161  
pregnanetriolone assay and, 172, 176, 179-180  
Permanganate, adrenaline assay and, 342-343  
Permutit, iodochrome separation on, 342  
Perri, method of, 299  
Peterson, method, 323-325  
Peterson and Pierce, method of, 65-67  
Petroleum ether, *see also* Light petroleum, Ligroin  
    adrenocorticosteroid isolation and, 213, 232, 239, 248, 249, 253  
    aldosterone isolation and, 292, 295, 296, 308, 309, 311, 317, 325, 328  
    estrogen separation and, 11, 13, 46  
    ketosteroid isolation and, 56, 60, 61, 66  
    pregnanediol isolation and, 156-157  
    pregnanetriolone isolation and, 177  
    progesterone isolation and, 97-98, 100-101, 124  
Pettenkofer reaction, ketosteroids and, 57  
*o*-Phenanthroline monohydrate, iodine determination and, 372  
Phenol red, iodoamino acid separation and, 362-363  
Phenolsulfatases, estrogen conjugates and, 6, 32  
Phenyl Cellosolve,  
    ketosteroid separation and, 74-76  
    progesterone isolation and, 106  
*m*-Phenylenediamine, ethanol purification and, 69, 222  
*o*-Phenylenediamine,  
    catechols and, 341  
    iodine determination and, 372  
Phenylhydrazine, adrenocorticosteroids and, 215, 217, 231-240  
Phospholipids,  
    aldosterone isolation and, 294  
    precipitation of, 92  
Phosphomolybdic acid,  
    adrenocorticosteroids and, 218  
    pregnanediol assay and, 158  
    progesterone identification and, 115-116  
Phosphoric acid,  
    adrenocorticosteroids and, 215, 250-251  
    aldosterone and, 287, 309  
    estrogen estimation by, 20  
    pregnanetriolone and, 170, 171, 174, 177-179  
Pincus and Pearlman, method of, 86  
Placenta, 2, 22  
    adrenocorticosteroids in, 207  
    enzyme preparation from, 61-62  
    estrogens in, 43  
    pregnanediol and, 164  
    progesterone in, 98, 119-121, 130-131, 143  
Plasma, *see also* Blood  
    adrenocorticosteroid conjugates in, 206-207, 253-254  
    catechol amines in, 347  
    collection of, 54  
    dehydroepiandrosterone in, 54-56  
    estrogens in, 41-44  
    hydrolysis of, 54-55  
    ketosteroids in, 80-84  
    progesterone in, 99, 119-121, 132-133  
    storage of, 5, 204  
    testosterone in, 60-63  
Polarography,  
    adrenocorticosteroids and, 256  
    estrogens and, 19  
    iodoamino acids and, 379-380  
    ketosteroids and, 88  
    progesterone determination by, 93, 107-108  
Porter and Silber,  
    method, 231-240  
    aldosterone and, 281, 287-290  
Potassium,  
    radioactive, aldosterone assay and, 268, 272-273  
Potassium *tert*-butoxide,  
    adrenocorticosteroid assay and, 252  
    aldosterone assay and, 278, 299  
    progesterone estimation and, 111  
Potassium hydroxide, stable solution of, 70-71  
Prealbumin, iodoamino acids and, 355  
Prednisone, tetrahydroaldosterone excretion and, 314  
Pregna-4,11-diene-3,20-dione, partition coefficients of, 100  
Pregnancy, 2  
    adrenocorticosteroids and, 203

- aldosterone in, 304, 317, 319
- cortisol in, 316
- estrogens in, 41
- 17 $\alpha$ -hydroxycorticoids in, 242
- pregnanediol and, 146
- pregnanetriolone and, 171-172, 181, 182
- progesterone in, 96, 131-133
- Pregnanediol, 170
  - adrenal studies and, 164-165
  - assay, 92-93
    - chromatography and, 149-150, 154-155, 158-159
    - enzyme hydrolysis and, 149, 154
    - future developments in, 165-167
    - gravimetric method, 147-148
    - hydrolysis and precipitation, 148, 154
    - refinements of, 149
    - sulfuric acid method, 148-149, 155, 159
    - recommended methods, 156-159
  - fertility and, 163
  - isolation of, 147
  - isomers of, 141
  - menstrual cycle and, 161-164
  - metabolic precursors, 140-141
    - organs producing, 142-144
  - occurrence of, 141, 144-145
  - paper chromatography of, 150
  - placental studies and, 164
  - precipitation of, 154
- Pregnanediol diacetate, chromatography of, 156-157
- Pregnanediol glucuronoside,
  - hydrolysis of, 153-154
  - isolation, assay and, 153
- Pregnane-3 $\alpha$ ,17 $\alpha$ -diol-20-one, occurrence of, 142
- Pregnanedione, formation of, 141
- Pregnan-17 $\alpha$ -ol-3,20-dione, pregnanetriol and, 142
- 5 $\beta$ -Pregnan-3 $\alpha$ -ol-20-one, occurrence of, 141
- Pregnanetetrol,
  - assay of, 227
  - occurrence of, 186, 188
- 5 $\beta$ -Pregnane-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,20 $\alpha$ -tetrol,
  - fluorescence of, 174
  - occurrence of, 170
- Pregnanetriol, 170
  - adrenal function and, 165
  - adrenocorticosteroid assay and, 230
  - adrenocorticotrophic hormone and, 181
  - assay, 150-151, 159-161, 172, 226-227, 251
    - accuracy of, 152
    - convenience of, 153
    - precision of, 152
    - sensitivity of, 152-153
    - specificity of, 151-152
    - future developments in, 165-167
  - chromatography of, 151, 154-155
  - color reactions of, 155-156
  - Cushing's syndrome and, 189, 190
  - fluorescence of, 177-179
  - isolation of, 150
  - metabolic precursors, 140-142, 194
    - organs producing, 142-144
  - occurrence of, 145, 182
  - stability of, 150, 154
  - virilism and, 143, 144, 192-193
- Pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ -triol, estimation of, 172
- 5 $\alpha$ -Pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol, fluorescence of, 174
- 5 $\beta$ -Pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol, fluorescence of, 174
- 5 $\beta$ -Pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ -triol, fluorescence of, 174
- Pregnanetriol glucuronoside, hydrolysis of, 154, 159, 160
- Pregnanetriolone,
  - adrenal cancer and, 171-172, 182-186
  - adrenal hyperplasia and, 171, 186-189, 251
  - assay, 227, 251
    - analytical procedure, 172-180
    - general considerations, 171-172
  - biosynthesis of, 193-196
  - fluorescence of, 174
  - occurrence of, 180-193
  - paper chromatography of, 173-176
  - virilism and, 192-193
- 5 $\beta$ -Pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol-11-one, significance of, 170
- Pregnanolone, isomers of, 141
- 5 $\beta$ -Pregn-5-ene-3 $\beta$ ,17 $\alpha$ ,20 $\alpha$ -triol, fluorescence of, 174
- Pregnenolone,
  - corticosteroids and, 143
  - ketosteroids and, 59



- separation of, 109
- Progesterone,  
  additional identification of, 115-118\*  
  aldosterone assay and, 278  
  bioassay of, 92, 93, 115, 119  
  chromatography, 93-98  
    column, 101-103  
    paper, 103-106, 119, 124-125, 127-129  
  corticosteroids and, 143  
  countercurrent distribution of, 93-95, 100-101, 113-114  
  elution from paper, 125, 126, 129  
  estimation, 94-95, 166  
    accuracy of, 119-122  
    applications of, 130-133  
    comparison of methods, 123  
    correction of, 112-115  
    derivatives and, 108-110  
    fluorescence and, 111-112  
    further developments in, 133-134  
    polarography and, 107-108  
    precision of, 122  
    recommended methods, 130  
    sensitivity of, 122-123  
    specificity of, 118-119  
    sulfuric acid-ethanol and, 111, 129-130  
    ultraviolet absorption and, 106-107, 125, 130  
  extraction of, 94-99  
  infrared absorption of, 117-118  
  isolation of, 92, 94-95, 100-106  
  partition coefficients of, 100, 102  
  pregnanediol and, 140-141  
  preliminary purification of, 94-95, 99-100  
  radioactive, 122, 124, 128  
  ultraviolet absorption, 92, 94-96, 119  
    correction of, 106-107  
  urinary pregnanediol and, 146
- Propanol,  
  iodoamino acid separation and, 362  
  progesterone isolation and, 104
- Propylene glycol,  
  aldosterone isolation and, 288-292, 296, 297, 308-310, 312  
  ketosteroid separation and, 61, 74-77  
  pregnanetriolone estimation and, 175-176
- progesterone isolation and, 104, 105, 128, 129
- Propylthiouracil, iodine determination and, 373
- Proteins,  
  adrenocorticosteroids and, 209  
  aldosterone and, 316, 327  
  estrogens and, 3-4, 43-45  
  hydrolysis, iodoamino acids and, 368-369  
  iodinated, 379  
    analysis of, 369  
    precipitation of, 358  
  Protein-bound iodine, significance of, 357-358
- Pseudohermaphroditism,  
  pregnanetriolone and, 170, 171, 181  
  urinary steroids and, 187
- Pseudomonas testosteroni*, hydroxysteroid dehydrogenases of, 21
- Pyridine,  
  digitonides and, 87  
  formazan elution by, 277
- Quenching,  
  catechol amine assay and, 344-345  
  cause of, 247
- Quinine, adreno-corticosteroid assay and, 232
- Quinol, estrogen estimation and, 19
- R**
- Raesside and Turner, method of, 119, 120
- Rat, aldosterone assay and, 267-269, 274-275
- Red cells,  
  estrogens in, 43-44  
  triiodothyronine uptake by, 356-357
- Reddy, method of, 236-238
- Reich, method of, 309, 329
- Renal function, aldosterone metabolites and, 320-322
- Resorcyllaldehyde, ketosteroids and, 60
- Rhodizonic acid, estrogen sulfates and, 34
- Rubin, method of, 73-78
- S**
- Saccharate, glucuronidase and, 206
- Saier, method of, 80-83

- Salicyloyl hydrazide, aldosterone assay and, 281-282
- Sandell and Kolthoff, method of, 370-375
- Schmidt and Staudinger, method, application of Dyrenfurth and Venning, 295-296
- Semen, estrogens in, 44
- Serum, *see* Blood, Plasma
- Short, method of, 119, 121, 127-128
- Silber, method, Moncloa modification of, 253-254
- Silber and Busch, method of, 239-240
- Silicic acid, adrenocorticosteroid isolation on, 213-214, 248 aldosterone isolation and, 294-295, 299, 308-311, 317, 328 estrogen separation on, 12-13 ketosteroid chromatography on, 55-56 pregnanediol isolation and, 159
- Silicone grease, estrogen separation on, 18
- Silicone paper, progesterone isolation and, 104
- Silver, iodine determination and, 370-371
- Simmer and Simmer, method of, 121
- Simpson and Tait, method of, 268, 271
- Singer, method of, 308, 329
- Singer and Stack-Dunne, method of, 308, 329
- Singer and Venning, method of, 267-268
- Skelly Solve, *see* Petroleum ether
- Sobel, method of, 310
- Sodium, radioactive, aldosterone and, 267, 272-273
- Sodium borohydride, adrenocorticosteroids and, 217, 234, 238, 241-245
- Sodium hydroxide, adrenocorticosteroid isolation and, 212 iodoamino acid separation and, 361
- Sommerville and Deshpande, method of, 119, 121
- Spirolactones, aldosterone and, 275
- Spleen, glucuronidase of, 205, 206
- Stachenko and Giroud, method of, 308, 329
- Starch, iodine determination and, 370
- Stein-Leventhal syndrome, pregnanetriolone in, 193 urinary steroids in, 164
- Stormshak, method of, 121
- SU4885, tetrahydroaldosterone excretion and, 314
- Sugars, adrenocorticosteroid assay and, 220
- Sulfadiazine, adrenocorticosteroid assay and, 232
- Sulfamerazine, adrenocorticosteroid assay and, 232
- Sulfanilic acid, iodine determination and, 372
- Sulfatase, thyroxine conjugates and, 365
- Sulfuric acid, adrenocorticosteroids and, 215, 248-250, 253 aldosterone and, 287, 290 catechol amine assay and, 344 estrogen estimation and, 20, 34, 37, 46 ketosteroid determination and, 58, 60 pregnanediol assay and, 148-149, 155, 157, 159 pregnanetriol and, 151, 155, 160 • progesterone estimation and, 95, 111, 116-117, 119 thyroxine and, 365
- Sulfuric-dichromate, iodine determination and, 359
- Sulfuric-permanganate, iodine determination and, 359

## T

- Tait, methods of, 325-327
- Talbot, method of, 85-86
- Talbot and Eitington, method of, 227-229
- Teridax, protein and, 357
- Testis, 2 ketosteroids and, 59
- Testosterone, 170 daily secretion of, 52-53, 59 determination of, 60-63 metabolites of, 52, 59 partition coefficients of, 100 separation of, 105 virilism and, 53

- Testosterone glucosiduronate, solvent partition of, 31
- Tetrabromothyronine, 377
- Tetrahydroaldosterone, 290
- chromatography of, 296, 315-316
  - estimation of, 277, 281, 303, 314-316, 322
  - excretion of, 320
  - extraction of, 294
  - identification of, 293
  - partition coefficient of, 316
  - properties of, 302
- Tetrahydrocortisol, 170, 200, 303,
- alkali and, 212
  - partition coefficient of, 316
  - phenylhydrazine and, 233-235
- Tetrahydrocortisone, 200, 255, 303
- partition coefficient of, 316
  - phenylhydrazine and, 235
- Tetrahydrocortisone monoglucosiduronate, recovery of, 207
- Tetrahydrofuran,
- deproteinization by, 54
  - formazan elution by, 277
- 3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ ,21-Tetrahydroxy-5 $\beta$ -pregnan-11-one, *see* Cortolone
- 3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ ,21-Tetrahydroxy-5 $\beta$ -pregnan-11-one, *see*  $\beta$ -Cortolone
- Tetraiodophenolphthalein, protein and, 357
- Tetraiodothyroacetic acid, paper chromatography of, 365, 366
- Tetraiodothyroacrylic acid, paper chromatography of, 366
- Tetraiodothyroformic acid, paper chromatography of, 366
- Tetraiodothyropropionic acid, paper chromatography of, 366
- Tetramethylammonium hydroxide,
- adrenocorticosteroid assay and, 219, 220, 222, 223
  - aldosterone assay and, 277
- Tetrazolium salts, *see also* Blue tetrazolium
- progesterone identification and, 116
- Thiocyanate, iodine determination and, 375
- Thiosemicarbazide, progesterone derivative, 94, 95, 108-109, 117, 119, 126-127, 134
- Thiosulfate,
- adrenaline assay and, 342, 346-347
  - iodine determination and, 352, 370
- Thiouracil,
- iodine determination and, 373
  - iodotyrosine chromatography and, 364
  - thyroxine extraction and, 360
- Thiourca, iodotyrosine chromatography and, 364
- Thymol, urine storage and, 204
- Thymol blue, iodoamino acid separation and, 362-363
- Thyroglobulin,
- column chromatography of, 362
  - iodine and, 379
  - isolation of, 367-368
  - ultraviolet spectroscopy of, 378-379
- Thyroid gland,
- desiccated, analysis of, 380
  - iodine in, 357
- Thyronine,
- paper chromatography of, 366
  - ultraviolet spectroscopy of, 379
- Thyroxamine, paper chromatography of, 366
- Thyroxine,
- arsenite-cerine reaction and, 370-371, 373-374
  - assay,
    - chemical methods, 369-378
    - general considerations, 353-354
    - historical, 351-353
    - iodo-compound separation and, 354 369
    - physical methods, 378-382  - column chromatography of, 361-363
  - conjugates of, 365
  - diazotized diethylsulfanilamide and, 378
  - extraction of, 359-360
  - formation of, 352
  - iodide formation from, 360-361
  - metabolic products of, 365, 373
  - ninhydrin and, 375-376
  - nitrous acid and, 376
  - paper chromatography of, 363-367
  - Pauly's reagent and, 377
  - plasma proteins and, 355-356
  - polarography of, 379-380
  - radioactive,

- binding of, 356
  - metabolism of, 380, 381
  - structure of, 352, 353
  - ultraviolet spectroscopy of, 378-379
  - Thyroxine glucuronide, paper chromatography of, 366
  - Tissues,
    - estrogens in, 43-44
    - storage of, 204
    - thyroxine binding in, 356
  - Toluene,
    - aldosterone isolation and, 286, 288-292, 296, 297, 308-310, 312, 325
    - estrogen isolation and, 8, 11, 33, 45, 46
    - noradrenaline assay and, 343
    - pregnanediol isolation and, 148, 149, 154, 156-158
    - urine storage and, 204
  - 3,5,6-Triacetoxy-N-methylindole, formation of, 339
  - Triacetyloleandomycin, adrenocorticosteroid assay and, 232
  - Trichloroacetic acid,
    - adrenocorticosteroid assay and, 244
    - 246
    - pregnanetriol and, 151
    - protein precipitation by, 358
  - Trihydroxyindole, catechol amine assay and, 344-345
  - 3 $\alpha$ ,11 $\beta$ ,21-Trihydroxy-5 $\beta$ -pregnan-20-one-18-al, *see* Tetrahydroaldosterone
  - Trihydroxy-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -pregnan-11-one, *see* Pregnanetriolone
  - 3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ -Trihydroxy-5 $\beta$ -pregnan-11-one, fluorescence of, 174
  - 3 $\alpha$ ,18,21-Trihydroxy-5 $\beta$ -pregnene-11,20-dione, 290
    - excretion of, 301-302, 316
  - 17,20,21-Trihydroxysteroids, assay of, 225, 228
  - 3,5,3'-Triiodothyroacetic acid, paper chromatography of, 366
  - Triiodothyronine(s),
    - column chromatography of, 361-363
    - desiccated thyroid and, 380
    - diazotized diethylsulfanilamide and, 378
    - ninhydrin and, 375-376
    - nitrous acid and, 376
    - uptake, red cells and, 356-357
  - 3,5,3'-Triiodothyronine,
    - arsenite-ceric reaction and, 373-374
    - discovery of, 353
    - paper chromatography of, 363-367
    - plasma proteins and, 355-356
    - ultraviolet spectroscopy of, 379
  - 3,3',5'-Triiodothyronine,
    - desiccated thyroid and, 380
    - occurrence of, 353
    - paper chromatography of, 365-366
  - 3,5,3'-Triiodothyropropionic acid, paper chromatography of, 366
  - 3,3',5'-Triiodothyropropionic acid, paper chromatography of, 366
  - Trimethylaminoacetohydrazide chloride, ketosteroids and, 85
  - Trishydroxymethyl aminomethane, iodoamino acid electrophoresis and, 355
  - Tritium, estrogens and, 22, 39-40
  - Trypsin, iodoamino acid liberation and, 369
  - Tryptophan, iodine determination and, 373
  - Tumor, iodoproteins in, 368
  - Turnbull's blue, estrogen estimation and, 19
  - Tyrosine,
    - iodine determination and, 373
    - paper chromatography of, 366
- U**
- Ulick, method of, 314-315, 322
  - Ultraviolet absorption,
    - aldosterone assay and, 276, 308-310
    - estrogens and, 19
    - iodoamino acids and, 378-379
  - Urea, adrenocorticosteroid assay and, 241
  - Urease, urine collection and, 69
  - Urine,
    - aldosterone-3-oxo conjugate in, 305-313
    - collection of, 67-69, 202, 204
    - dehydroepiandrosterone in, 53-54, 56-59
    - estrogen estimation in, 34-41
    - extracts, 68, 69
      - chromatography of, 75-76
    - hydrolysis, 68, 69
      - aldosterone assay and, 305-307

17-ketosteroids in, 63-72  
pregnanediol in, 145, 156-159  
pregnanetriol in, 145, 159-161  
pregnanetriolone in, 173-180  
storage of, 4-5

Urokon, protein and, 357

Uterus, progesterone in, 130

## V

Venning, method of, 271, 272

Vestergaard, method of, 67

Virilism,

adrenal and, 144

ketosteroids and, 53, 59, 63, 170, 192-193

ovarian hormones and, 142-143

urinary steroids in, 183, 191-193

## W

Water, progesterone isolation and, 101

Waxes, adrenocorticosteroid assay and, 220

## Y

Yeast, adrenocorticosteroid assay and, 240

## Z

Zander, method of, 120

Zander and Simmer, method of, 119, 123-127

Zimmermann reaction, *see also m*-Dinitrobenzene

adrenocorticosteroids and, 215, 225, 228, 240

aldosterone and, 287

interfering substances and, 243

ketosteroids and, 56, 64, 65, 72, 75, 77, 80, 83

methods of, 84-85

pregnanetriol and, 151

Zinc-acetic acid, adrenocorticosteroid assay and, 242

Zinc hydroxide, protein precipitation and, 358

Zondek and Finkelstein, method of, 177-179